



Control of nighttime transpiration and its effects on water-use efficiency in grapevine

Adrianus J Westgeest

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Control of nighttime transpiration and its effects on water-use
efficiency in grapevine

Déterminismes de la transpiration nocturne et ses effets sur
l'efficience d'utilisation de l'eau chez la vigne

Adrianus J. Westgeest
Le 18 Décembre 2020

Sous la direction de Florent Pantin
et Thierry Simonneau

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Adrianus J. Westgeest

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Chapter 1

General Introduction

Background

Almost 300 years ago, the pioneering plant physiologist Stephen Hales performed the first experiments to elucidate the mystery of plant water consumption (Hales et al., 1727). Initially, it was thought that water was cycling in the plant just as blood in humans. Rather, he demonstrated the upward motion of water from roots to the shoot. He added a quantity of water to a potted sunflower, covered the soil and weighted the pot to conclude that water must be lost at the interface of the leaves with the atmosphere. In his theory, he proposed that small pores in the leaf were responsible for the 'perspiration', better known nowadays as transpiration. Microscopist Marcello Malpighi had already described these pores called stomata in *Anatomia plantarum* (1671) but had no idea about their function. In the following century, the role of stomata was unravelled. Against all common belief at that time, plants appeared to nourish themselves from the air, specifically carbon dioxide (CO₂), to create the building blocks for plant growth (Hill, 2012). The photosynthesis is grounding this process which uses sunlight to transform CO₂ by creating chemical bounds. The stomatal pores are essential for photosynthesis to happen, allowing the diffusion of CO₂ from the air into the leaf, while water is simultaneously lost to the atmosphere.

Yet people were not convinced about the power of stomata to control plant water losses. The transpiration rate of plants has long been thought to be essentially dependent on meteorological variables (Jarvis and Mcnaughton, 1986). Good correlations between field evaporation data and weather conditions led to the opinion that physical rather than physiological factors regulated transpiration. Plants were only 'wet' and only passive conductors of water. However, two observations did conflict with this theory: leaves are not a 'wet' surface but covered by a waxy layer and stomatal pores can open or close depending on the environment (Darwin, 1898). Around 50 years ago, the idea of the existence of an important canopy resistance to transpiration started to dawn with stomata as the most important regulator of water losses (Monteith et al., 1965).

Nowadays, stomata are thought to be essential for terrestrialization of life on earth. The colonization of the dry land by early plants 500 Mya required fundamental adaptations to cope with lower water abundance and dry atmosphere (Raven, 2002). Leaves need to avoid dehydration while at the same time maximizing gas entry of CO₂ for photosynthesis. Tissue dehydration was prevented by the development of a waxy layer covering the leaf called the cuticle (Kong et al., 2020; Renault et al., 2017), and CO₂ entry was facilitated by pores in the cuticle, called stomata (Berry et al., 2010; Chater et al., 2017). The opening of these pores for CO₂ diffusion inevitably results in water losses. Accordingly, complex signalling pathways evolved to maximize carbon gain per unit water loss (Brodribb and McAdam, 2017; Sussmilch et al., 2019). Understanding the stomatal regulation of transpiration is a large research field as it may help to improve plant water use, which became extremely important nowadays to battle water scarcity in agriculture.

Towards plants with a more efficient control of transpiration

The intimate relationship between transpiration and photosynthesis indicates that water supply is essential to sustain plant growth. Opening of stomatal pores enhance photosynthesis by facilitating CO₂ diffusion into the leaf but at the expense of water vapour lost to the atmosphere. Low water availability in leaves closes stomata to avoid further dehydration of the leaf. As a result, CO₂ diffusion is hindered reducing photosynthesis and growth. Avoiding the disastrous effects of water deficits on plant productivity makes agriculture the largest consumer of freshwater in the world (Cominelli et al., 2009). This does not avoid that drought causes the biggest yield losses in agriculture (Gupta et al., 2020). The demand for water for food production is predicted to double in the future while water availability is being decreased by climate change and the needs of a growing human population

(Falkenmark, 2013), urging the need to use water more efficiently in agriculture (Morison et al., 2008). Plants show a tremendous variation in the amount of water needed for growth, and understanding the water use of plants can help to produce high-yielding, water-use efficient crops.

Water-use efficiency

A key process to increase water-use efficiency of crops is to acquire more carbon in exchange for less water transpired by the crop (Condon et al., 2004). The amount of carbon fixed per unit water can be calculated instantaneously by dividing carbon assimilation rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$) by the rate of water loss ($\text{mmol m}^{-2} \text{s}^{-1}$) at a given point in time that represents the intrinsic water-use efficiency (WUE_i , Yoo et al., 2009). When stomata open in response to light, water is lost depending on the difference between inside and outside vapour pressures of water (vapour pressure difference from leaf to air, VPD) and the resistances to water vapour transfer along this gradient (Farquhar and Sharkey, 1982). These resistances to water flow at the leaf surface include stomata, cuticle (the waxy layer surrounding the epidermis) and the boundary layer (air layer adjacent to the leaf where wind speed is reduced by friction). Stomatal opening at light is linearly related to transpiration if we assume that VPD is constant between the leaf and the air (Figure 1). Depending on the environmental conditions, this may not be completely correct. Especially in low-wind speed conditions, transpiration is expected to humidify the boundary layer surrounding the leaf, increasing its resistance. In normal outdoor conditions the boundary layer effect is decreased by wind and thought to play a minor role on controlling transpiration. Moreover, transpiration decreases the temperature of the leaf changing the internal saturated vapour pressure, thereby reducing the VPD. However, leaf temperature can be maintained nearly constant by the continuous warming of the leaf by light energy and dehydration of the intracellular spaces if not rehydrated directly (Buckley et al., 2017). All these conditions supports that stomatal conductance can be considered as linearly related to transpiration in outdoor conditions. CO_2 assimilation increases linearly with stomatal conductance rises within a range of low values, but saturates at high stomatal opening (Figure 1). This limitation is caused by the saturating activity of the photosynthesis-related enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco, Flexas et al., 2010; Thompson et al., 2007). So, photosynthesis may be limited by low values of stomatal conductance but not anymore at high levels of stomatal conductance. The CO_2 assimilation rate can be modulated by the mesophyll conductance or by the carboxylation efficiency of photosynthetic enzymes which may move the maximal WUE towards higher rates of CO_2 assimilation (Condon, 2020). Hence, maximal WUE is obtained by reducing excessive stomatal apertures, avoiding the saturation of CO_2 assimilation (Figure 1). However this reduces the CO_2 assimilation and therefore often higher WUE efficiency is coupled to a lower production (Blum, 2005; Condon et al., 2002). So, due to the coupling between water and CO_2 flows decreasing water losses during the day has direct impacts on photosynthesis and plant growth. However, the WUE presented here does not take into account the water losses in the absence of photosynthesis, which penalizes the WUE.

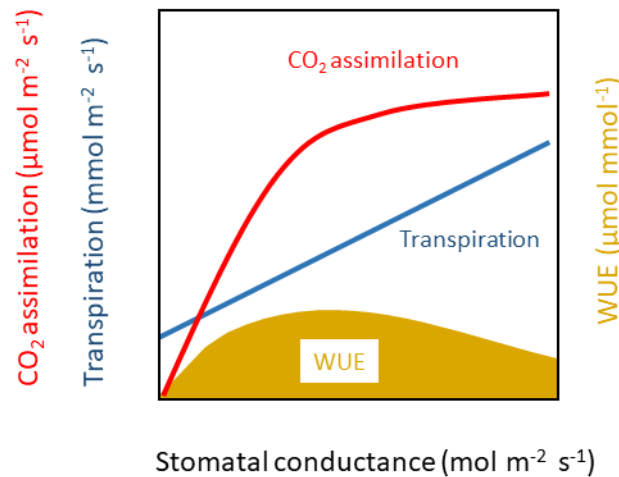


Figure 1: Relationships between stomatal conductance and transpiration, CO₂ assimilation and water-use efficiency (WUE). Transpiration rate increases linearly with stomatal conductance, whereas CO₂ assimilation rate saturates at high stomatal conductance. WUE increases when the slope of CO₂ assimilation is higher than the increase in transpiration and decreases at high stomatal conductance. Figure adapted from Yoo et al. (2009).

Integrating nighttime water losses in water-use efficiency

At night, there is no need for carbon dioxide and stomata close to avoid inefficient water losses (Caird et al., 2007). The residual transpiration levels at night differ considerably among and within plant species (Christman et al., 2008; Daley and Phillips, 2006; Marks and Lechowicz, 2007; Ogle et al., 2012) and in different environmental conditions or habitats (Barbour and Buckley, 2007; Marks and Lechowicz, 2007). It is generally thought that incomplete stomatal closure is responsible for the water losses at night (Caird et al., 2007). This residual stomatal opening leads to transpiration rates that can be as high as 30 % of daytime rates observed in many species in the field (Benyon, 1999; Bucci et al., 2004; Snyder et al., 2003). Intuitively these water losses are inefficient as it is not directly related to CO₂-fixation. Alternative functions have been proposed to understand the potential benefit that could outweigh the disadvantage of water losses at night. Having a higher transpiration at night could possibly favour nutrient supply for growth at night, but did not have any effect in *Arabidopsis thaliana* (Christman et al., 2009). Also, plants of *Helianthus annuus* and *Populus* did not adapt their nighttime transpiration in nutrient-limited environments (Howard and Donovan, 2007, 2010). Alternatively, higher stomatal opening at night could be favour reopening at light on the following morning thereby slightly increasing the subsequent photosynthesis. However only minimal differences in photosynthesis could be observed which does not reinforce this hypothesis (Auchincloss et al., 2014). In the absence of a potential beneficial effect of nighttime transpiration, negative effects have been found for high levels nighttime transpiration. Nocturnal water losses slowed down the rehydration of tissues at night in trees (Bucci et al., 2004; Kavanagh et al., 2007). In grapevine, high nighttime water losses are found in cultivars known to have a 'optimistic' behaviour to use water, known as anisohydry (Rogiers et al., 2009). Moreover, high nocturnal water losses penalized the WUE among grapevine genotypes from a biparental cross between Syrah and Grenache (Coupel-Ledru et al., 2016). It is thus tempting to select genotypes with low nighttime transpiration as they likely use water with higher efficiency. However, a positive correlation is usually found between daytime and nighttime water losses (Christman et al., 2008; Coupel-Ledru et al., 2016), raising the risk of reducing daytime photosynthesis at the same time of saving water by selecting for lower nighttime transpiration. This

relation could be genetically uncoupled as quantitative trait loci (QTL) were found both for lower nighttime transpiration and increased biomass (Coupel-Ledru et al., 2016). This strongly suggests that breeding for lower nighttime transpiration can increase the WUE of plants without negative undesired effects on growth. In the same study, both the number of stomata on the leaf and incomplete stomatal closure were proposed as potential actors underlying this QTL for nighttime transpiration and higher production of biomass.

Stomatal control of water losses

Stomatal density

Stomata consist of two identical guard cells which originate from a stomatal precursor cell: the meristemoid (Bergmann and Sack, 2007). The number of stomata on a leaf is the ultimate result of the initial number of meristemoids and the frequency of cells divisions it undergoes. Many early acting genes have been characterised in mutants of *Arabidopsis* which are responsible for the correct patterning and spacing of at least one pavement cell between stomata (Han and Torii, 2016). Genetic manipulation of these genes in multiple plant species has led to the development of genotypes exhibiting lower stomatal density (Caine et al., 2019; Hepworth et al., 2015; Hughes et al., 2017; Masle et al., 2005; Tanaka et al., 2013; Yoo et al., 2010). Indeed, lower stomatal density decreased transpiration thereby improving WUE or drought tolerance, however in most cases this was associated with smaller plant size or slower growth. Nevertheless, this can provoke a 'conservative' behaviour of water use which may be advantageous during water stress (Buckley et al., 2020). In the case of incomplete stomatal closure at night, the density of such 'leaky' stomata will determine the residual flux of water (Tominaga et al., 2020). However, higher stomatal density entails smaller stomata (Franks and Beerling, 2009; Hetherington and Woodward, 2003), that respond faster to environmental cues (Drake et al., 2013; Lawson and Blatt, 2014). Moreover, increasing stomatal density may not increase transpiration as it can be accompanied with lower stomatal aperture (Büßis et al., 2006). So, the positive effect of stomatal density on nighttime transpiration through leaky stomata may be compensated by smaller, more reactive stomata and a general smaller aperture of the pore.

Stomatal opening

Stomata open and close via changes in the turgor pressure of the guard cells. At the origin of these changes, influxes of cations (K^+), anions (Cl^-) or sugars decreases the water potential and forces water to entry into the guard cell (Figure 2, Kollist et al., 2014; Lawson and Matthews, 2020). Water entry increases the turgor pressure causing the guard cell to swell up and bend. Bending of the guard cells is essential to create the pore opening and is the result of a the radial orientation of cellulose fibrils around the guard cell (Aylor et al., 1973). The opening of stomata is predominantly activated by light which activates two parallel signalling pathways. One is specific to blue light and the other is stimulated by the whole spectrum of photosynthetic active radiation (Roelfsema et al., 2006; Shimazaki et al., 2007). Blue light irradiation of guard cells activates the blue light-photoreceptor protein kinases, called phototropins (PHOT1 and PHOT2 (Kinoshita et al., 2001)), through autophosphorylation and initiate the signalling for stomatal opening (Inoue and Kinoshita, 2017). The activated phototropins phosphorylate the protein kinase BLUE LIGHT SIGNALING1 (BLUS1) (Figure 2, Takemiya and Shimazaki, 2016), and in turn generates a signal to activate the proton H^+ -ATPase pump (Cotelle and Leonhardt, 2016; Shimazaki et al., 2007). H^+ efflux results in the hyperpolarization of the membrane and activates the channels that transport K^+ and anions Cl^- , nitrate and malate²⁻ into the guard cell (Figure 2, Kim et al., 2010; Lebaudy et al., 2008a). The accumulating K^+ and Cl^- are directly transported into the vacuole via the tonoplast transporters (De Angeli et al., 2013). The influx of these anions and sugars drive the osmotic influx of water, and the increasing in volume and turgor cause the guard cell to swell and

create the opening of the stomatal pore. The pathway activated by blue light is known as the most effective way to open stomata in response to light (Kinoshita and Hayashi, 2011). The blue light response by phototropins also activates the breakdown of triacylglycerol to provide energy to the proton pumps (McLachlan et al., 2016). Blue light also activates starch degradation in guard cells which may provide osmolytes as malate and sugars, or ATP via cellular respiration to fuel H^+ -ATPase and stomatal opening (Horrer et al., 2016).

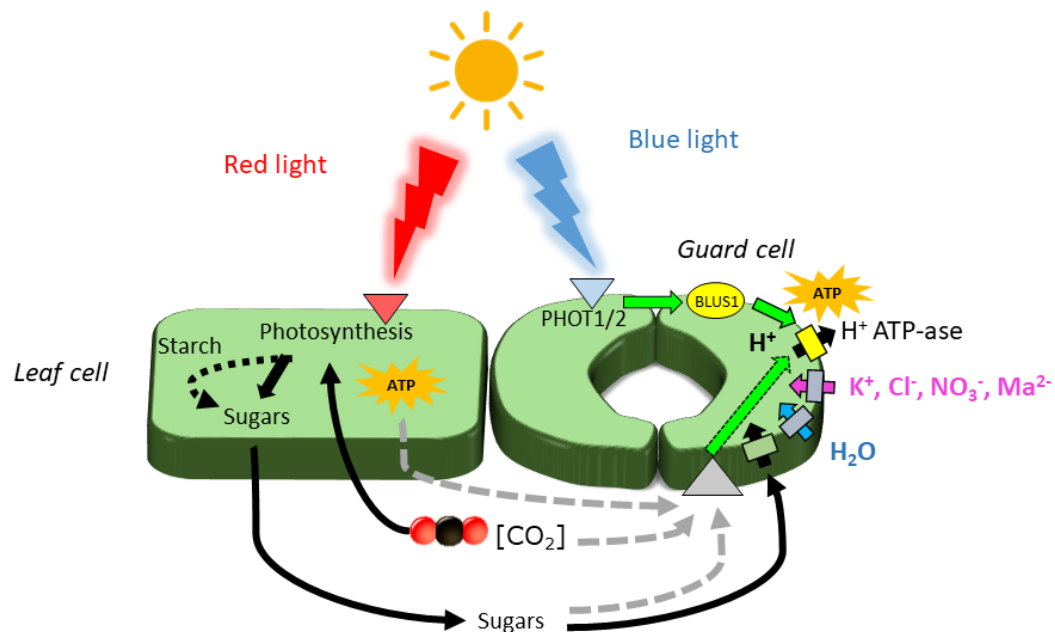


Figure 2: Pathways promoting stomatal opening. Light is grounding the proces of stomatal opening by two parallel signalling pathways. Blue light is sensed in the guard cells by the phototropins PHOT1 or PHOT2, phosphorylate BLUS1 to create a signal that activates the H^+ ATPase proton pump requiring ATP. The resulting efflux of H^+ hyperpolarizes the guard cell membrane. This results in the influx of K^+ , Cl^- , NO_3^- , Ma^{2-} and forces the osmotic influx of H_2O , increasing volume and swelling of the cell. The second process is involving red-light, the activation of photosynthesis in epidermal or mesophyll cells. The activation of photosynthesis may produce several signals in the substomatal apoplast sensed by the guard cells: the accumulation of ATP, decreasing CO_2 concentrations and the accumulation of sugars. Recently a guard-cell specific sugar importer of glucose was found to be important for light-induced stomatal opening. Dotted lines reflect a hypothetical pathway with yet unidentified actors. Grey lines imply a sensing mechanism of the guard cells. Green arrows represent an activation step.

Stomatal opening is strongly correlated with photosynthesis (Wong et al., 1979) which suggests that guard cell movements may be under the control of photosynthesis-derived signals. Indeed, stomatal mutants impaired in the blue-light opening response (*phot1phot2*) still open in response to light (Wang et al., 2020). This response is dependent on the photosynthesis as irradiation by red light induced stomatal opening only when the intensity was high enough to activate photosynthesis (Roelfsema et al., 2006). The nature of the signals and their origins are a matter of debate. Photosynthesis-related signals may be produced in the guard-cell itself or in the mesophyll. Stomata in albino patches of *Chlorophytum comosum* were responsive to blue light and CO_2 but not to red light (Roelfsema et al., 2006), suggesting that the photosynthesis in the mesophyll is needed for this response. Moreover, illumination of a single guard cell did not result in stomatal opening contrary to the illumination of the whole leaf (Roelfsema et al., 2006). The stomatal response to red-light could be mediated by an intracellular drop in CO_2 concentrations created by the use of CO_2 by the photosynthesis (Fig. 2).

Stomata are known to open in response to low CO₂ concentrations while they close when concentrations are high. This response is probably sensed by stomata in the intracellular space by yet an unknown signalling mechanism (Mott, 1988). It is attractive to think that both red-light responses and CO₂ responses are regulated by a common mechanism such as photosynthesis. A possible candidate related to photosynthesis for the signalling of CO₂ in stomata is the concentration of xanthophyll zeaxanthin in leaves which is dependent on the variation in pH with light reactions in the chloroplast and is linearly related to stomatal apertures (Zeiger and Zhu, 1998; Zhu et al., 1998). Alternatively, chloroplast ATP production from photophosphorylation could be the intermediate signal (Figure 2, Buckley et al., 2003; Tominaga et al., 2001). Transgenic alteration of photosynthesis was performed to check if and how photosynthesis may regulate stomatal opening. When the activity of Rubisco or cytochromes was severely reduced by transgenesis, photosynthesis decreased in both guard cells and in the mesophyll but did not impact stomatal conductance in *Nicotiana tabacum* (Baroli et al., 2008; von Caemmerer et al., 2004). Contrastingly, stomatal responses to light were increased when mutating the Calvin cycle enzyme sedoheptulose-1,7-bisphosphatase (SBPase) which decreased quantum efficiency of photosystem II electron transport in both guard and mesophyll cells (Lawson et al., 2008). Following this study, it was proposed that products of the photosystems accumulated directly impacting stomatal movements. High ambient extracellular CO₂-concentrations led to the accumulation of malate in the guard cell apoplast, potentially derived from mesophyll photosynthesis (Hedrich and Marten, 1993; Hedrich et al., 1994) and imported by ABC transporters resulting in stomatal closure (Lee et al., 2008). Recently, it was shown that guard cells contain several glucose importers that are important for stomatal opening in response to light (Figure 2, Flütsch et al., 2020). A similar glucose transporter was found in the subsidiary cells and promoted stomatal opening (Wang et al., 2019). This suggests that mesophyll-derived sugars are important for providing carbon sources to facilitate stomatal opening.

Increasing the stomatal conductance during the day by increasing stomatal density or opening may only be advantageous for the WUE if stomata are limiting photosynthesis (Figure 1). If this is the case, increasing stomatal conductance will increase photosynthesis and growth (Wang et al., 2014). Recently, optogenetic stomatal engineering of a blue-light controlled K⁺ channel increased stomatal conductance and the resulting increases in photosynthesis outweighed the higher water losses resulting in a higher WUE (Papanatsiou et al., 2019). These plants grew faster and showed a stronger stomatal closure at darkness. Exactly similar results were obtained by specific overexpression in guard cell of a circadian clock regulator (Hassidim et al., 2017). Hence, assuming that stomatal aperture is limiting photosynthesis, stronger light-induced opening may increase photosynthesis and WUE provided that nighttime stomatal closure is not impaired.

Stomatal closure

Stomatal closure involves the efflux of ions and water which deflates the guard cells and reduces stomatal aperture (Kim et al., 2010). The efflux of ions such as Cl⁻ and NO₃⁻ is facilitated by S-type anion channels (slow, as SLAC1, Figure 3, Hedrich and Geiger (2017). R-type anion channels are also activated and transport inter alia malate out of the cell (Meyer et al., 2010). The efflux of ions depolarizes the guard cell plasma membrane, enabling the efflux of K⁺ via voltage-dependent potassium channels such as KAT1 (Locascio et al., 2019). At the same time, the vacuolar membrane is depolarized through the activation of K⁺ selective TPK channels, which supports the efflux of anions through Aluminum-Activated Malate Transporters (ALMTs). The efflux or metabolization of solutes is accompanied by water and the guard cell deflates by a loss in turgor pressure (Penfield et al., 2012). The main activator of this process is abscisic acid (ABA) (Kollist et al., 2014; Raghavendra et al., 2010). ABA activates S and R type channels (Figure 3, Roelfsema et al., 2004) and inhibits the activity of the H⁺ ATPase (Goh et al.,

1996). The inhibition of the H^+ ATPase is facilitated by an *N*-ethylmaleimide-sensitive factor attachment protein receptor protein called VESICLE-ASSOCIATED MEMBRANE PROTEIN (VAMP711) (Xue et al., 2018). ABA is sensed by PYR/PYL/RCAR receptors. These receptors block 2C protein phosphates (PP2C) in order to activate OST1-mediated phosphorylation of S and R-type anion channels resulting in the efflux of ions (Geiger et al., 2009; Ma et al., 2009; Mustilli et al., 2002). Interestingly, loss of PYR/PYL/RCAR receptors increased transpiration three-fold compared to wild-type, indicating that stomatal apertures are strongly regulated by ABA in normal well-watered conditions (Gonzalez-Guzman et al., 2012; Merilo et al., 2013). OST1 also activates the production of reactive oxygen species (ROS), which activate Ca^{2+} -permeable channels (Figure 3, Kwak et al., 2003; Pei et al., 2000). The resulting boost of cytosolic Ca^{2+} leads to the activation of calcium-dependent protein kinases (CPKs) that in turn activate S and R-type anion channels (Geiger et al., 2010). The ABA-induced burst of ROS is dampened by autophagy (Yamauchi et al., 2019) and flavonols (Medeiros et al., 2020; Watkins et al., 2017). Higher flavonol concentration in the guard cells resulted in stronger stomatal opening and attenuated closure in response to ABA. Other external stomata-closing stimuli as the ROS hydrogen peroxide or strigolactones exploit CPKs to close stomata (Lv et al., 2018; Rodrigues et al., 2017; Sierla et al., 2016). As mentioned before, sugars may increase stomatal opening but they can also be involved in stomatal closure (Daloso et al., 2016). Both sucrose and glucose may trigger stomatal closure via phosphorylation by a hexokinase (HXK) (Figure 3, Kelly et al., 2013; Li et al., 2018; Lugassi et al., 2020). It is suggested that over accumulation of sucrose in the mesophyll may diffuse to the stomata to induce stomatal closure (Kelly et al., 2013). High CO_2 closes stomata and this response is mediated by two carbonic anhydrases at the guard cell membrane (Figure 3, Hu et al., 2010).

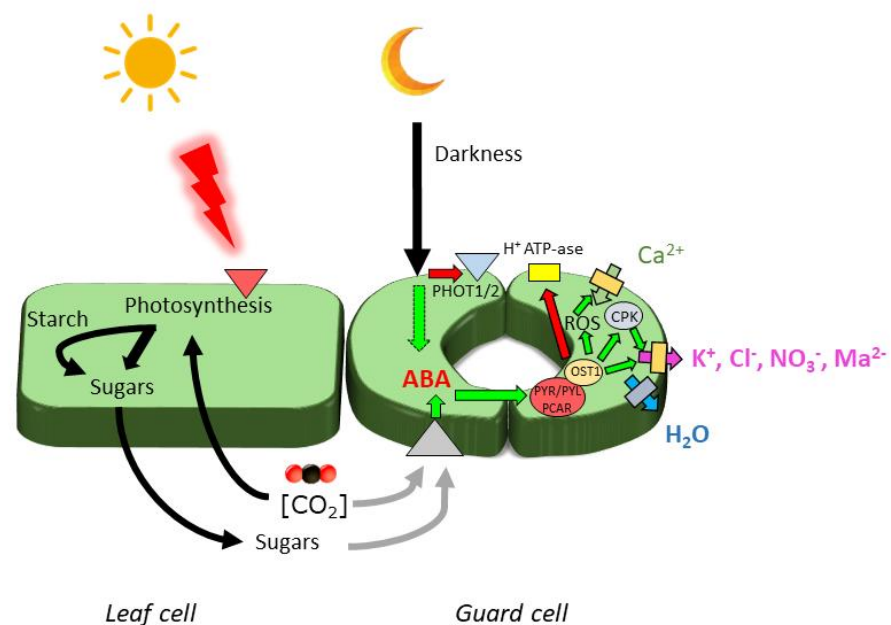


Figure 3: Pathways promoting stomatal closure. In the presence of stomata closing stimuli as darkness or photosynthetic activity. Firstly, darkness deactivates the PHOT1 pathway that activates H^+ -ATPase. Darkness also involves the ABA pathway activating protein kinase OST1 via the PYR/PYL/PCAR receptors. OST1 activates the S and R type channels exporting Cl^- , NO_3^- and Ma^{2-} . This efflux depolarizes the membrane forcing K^+ and subsequently H_2O to leave the cell. This reduces turgor, cell shrinking and closure of the pore. Stomatal closure also involves a burst of ROS that activates Ca^{2+} permeable channels. Increasing Ca^{2+} concentrations induce the CPK protein kinase activity that turns on S and R type channels. Sugars produced by adjacent photosynthetic cells may accumulate in the apoplast and be sensed by hexokinases at the guard cell membrane, stimulating stomatal closure which is mediated

by ABA. High CO₂ may also be sensed by the guard cell and induce stomatal closure. Dotted lines reflect a hypothetical pathway with yet unidentified actors. Grey lines imply a sensing mechanism of the guard cells. Green arrows represent an activation step while red arrows represent a desactivation.

Decreasing stomatal conductance may increase the WUE but is accompanied with negative effects on photosynthesis (Figure 1), and possibly less efficient evaporative cooling of the leaf surface during the day (Buckley et al., 2020). Nevertheless, many studies explored this strategy to improve WUE. This has been done by increasing the ABA-sensitivity of guard cells (Mega et al., 2019; Yang et al., 2016) or overstimulating the production of ABA (Thompson et al., 2007). Alternatively, mutation of a multidrug ABC-transporter in guard cells (Klein et al., 2003) or a cellulose synthase (Liang et al., 2010) and overexpression of a Photosystem II Subunit S protein in the chloroplast (Głowacka et al., 2018) also resulted in stronger stomatal closure and better WUE. These attempts could not avoid a delay in growth rate or development and can only be useful if simultaneously increasing the photosynthetic efficiency by increasing mesophyll conductance or carboxylation efficiency (Condon, 2020).

By targeting stomatal closure at night, the negative effects on growth can be circumvented together with a gain in WUE. Incomplete stomatal closure (hereafter called leaky stomata) has been proposed as the major factor of high water losses at night (Caird et al., 2007). Stomatal closure in response to darkness is attenuated in ABA-deficient plants, indicating that darkness-induced closure is at least partly dependent on ABA (Leymarie et al., 1998; Merilo et al., 2013; Sierla et al., 2018). Moreover, low concentrations of ABA have been attributed to slow stomatal closure to darkness in *Rosa x hybrida* (Terfa et al., 2020). Interestingly, actin filament reorganisation mutants did not show stomatal closure in response to darkness (Isner et al., 2017; Jiang et al., 2012) suggesting a role of actin filaments in the control of stomatal closure. However, these mutants did also not respond to light. Actin filaments could therefore simply control the geometry of guard cells in their resting state (minimal aperture).

Improving the coordination between carbon gains and water losses

Faster stomatal response

Ideally, stomatal opening should be synchronized with photosynthetic activity. The leaf is subjected to continuously fluctuating light due to clouds, sun flecks, shading from neighbouring plants, overlapping leaves with wind-driven movements (Lawson et al., 2010). Both stomata and photosynthesis respond to light cues, but these responses are not always synchronized as stomatal movements does not respond as rapidly as photosynthetic activity (Lawson and Blatt, 2014). The lag in stomatal response results in either waste of water in the absence of light or limitation of photosynthesis under light conditions. Nevertheless, continuous synchronization of stomata with photosynthesis may be costly in terms of energy and solutes requirements in the guard cells (Lawson and Vialat-Chabrand, 2019). Therefore, having faster stomatal responses may only be beneficial in a specific environment.

Diel endogenous control of stomatal movement

Stomata open and close following the 24-hours (diel) pattern of sunlight. During the day, stomatal aperture also intimately parallels the diurnal pattern of sunlight intensity: stomata gradually open until midday and slowly close in the afternoon (Matthews et al., 2018, 2019; Rogiers et al., 2009). To a lesser extent, this sinusoidal pattern is also observed in laboratory conditions with stable light intensity (Gorton et al., 1989; Kaiser and Kappen, 1997; Lanoue et al., 2018; Matthews et al., 2017; Webb, 2003), suggesting a light-independent control of diurnal stomatal behaviour. At night, stomata close, but tend to reopen in uncontrolled (Bucci et al., 2004; Donovan et al., 2003; Howard and Donovan, 2007, 2010;

Resco de Dios et al., 2016; Schwabe, 1952) and controlled conditions (Easlon and Richards, 2009; Hassidim et al., 2017; Resco de Dios et al., 2015). The dynamic behaviour of stomata in stable environmental conditions suggests that stomatal aperture is regulated by endogenous processes. Both diurnal and notably nocturnal stomatal patterns may potentially be costly for the plants water use as they are not coupled or well co-ordinated with carbon gains (Matthews et al., 2017, 2018).

Previous reports have suggested that diel patterns in stomatal aperture are initiated by circadian rhythms (Dodd et al., 2004; Hassidim et al., 2017; Hubbard and Webb, 2015; Resco de Dios et al., 2016). The circadian clock is a mechanism orchestrating a biological 24-hour rhythm to confer adaptive advantages in plants (Caldeira et al., 2014; Dodd et al., 2005; Green et al., 2002). The clock helps to optimize plant performance by anticipating to the predictable 24-h cycles caused by the rotation of the planet. Plants anticipation following the 24-h cycle allows a higher sensitivity to environmental stimuli depending on the time of the day (Joo et al., 2017; Mencuccini et al., 2000). The circadian clock controls ABA concentration and associated signalling and may thereby control stomatal opening (Legnaioli et al., 2009; Robertson et al., 2009). The key clock regulator TOC1 (TIMING TO CAB EXPRESSION 1) interacts with ABA signalling and is hypothesized to decrease/increase stomatal opening in the late afternoon/night (Pokhilko et al., 2013; Webb, 2003). This process may prevent wasteful use of water for CO₂ assimilation in the afternoon when evaporative demand is strongest and maximize CO₂ assimilation in the cool morning. Increases in stomatal aperture during the night may anticipate dawn to increase stomatal opening in response to light but not yet proven (Auchincloss et al., 2014). ABA also triggers ROS production which is peaking in the afternoon and may mediate stomatal closure (Li et al., 2020). So, the circadian clock may influence both day and nighttime stomatal patterns but the potential benefit for plant fitness or crop yield is not clear.

Photosynthesis-derived signals may also modulate stomatal aperture. During the day, photosynthesis-derived signals such as ATP (Tominaga et al., 2001) and glucose (Flütsch et al., 2020; Wang et al., 2019) may provide channel with activation energy or perform as osmoticum to increase turgor and induce stomatal opening. On the other hand, malate (Hedrich and Marten, 1993; Hedrich et al., 1994; Lee et al., 2008), sucrose (Kelly et al., 2013) and ROS (Iwai et al., 2019) may induce stomatal closure. The timing during the day, as well as how these signals are transported and accumulated will determine their action on stomatal aperture. Different light intensities and regimes can change CO₂ assimilation with a tremendous effect on the amplitude of the diurnal sinusoidal pattern (Matthews et al., 2018). It should be noted that the photosynthesis is also regulated by the circadian clock (Dodd et al., 2015; Hennessey and Field, 1991), and vice-versa the circadian clock oscillators adjust themselves with sugar signalling to create sugar homeostasis, avoiding carbon starvation (Seki et al., 2017).

Stomatal reopening at night

Part of the sugars produced by daytime photosynthesis is stored as starch to sustain growth at night. Starch breakdown at night results in the accumulation of sugars potentially inducing stomatal aperture. This hypothesis is comforted by the observation that higher daytime photosynthesis in *Vicia faba* increased the amplitude of a nighttime stomatal reopening pattern (Easlon and Richards, 2009). Moreover, the pattern was absent in a starch-deficient mutant in *Arabidopsis* called *pgm* (Lascève et al., 1997). However, growth at high CO₂ levels *Eucalyptus camaldulensis* did not increase the accumulation of starch but did show a stronger stomatal reopening pattern at night (Resco de Dios et al., 2016). This gave rise to an alternative hypothesis. It has been proposed that nighttime reopening patterns are the response of stomata to increasing intracellular CO₂ concentrations from nighttime respiration (Even et al., 2018; Fricke, 2019). Next to the active regulation of stomatal reopening, also passive regulation by hydraulic signals may influence stomatal aperture. Guard cell turgor may be decreased by low leaf water potential in the daytime and during soil drying (Tombesi et al., 2015), and

increased during rehydration (Buckley, 2005). The latter process is happening at night but is expected to play a minor role in well-watered conditions. Besides, disruption of inward K^+ channels in guard cells led to a reduction in the amplitude of nighttime stomatal reopening, suggesting that K^+ is the dominant osmoticum for increases in turgor at night (Lebaudy et al., 2008b).

So, the endogenous control of diel stomatal movements is modulated by several interconnected pathways such as the circadian clock and CO_2 assimilation. Variation in diel stomatal patterns have been described both between (Matthews et al., 2017) and within species (Resco de Dios et al., 2016) but the genetic controllers are not yet explored at large genetic scale.

Cuticular transpiration

The cuticle is a waxy layer covering all above-ground organs of the plant. Forming the barrier with the atmosphere, the cuticle prevents drying out of the leaf epidermal cells (Kerstiens, 1996; Kong et al., 2020; Riederer and Schreiber, 2001). General consensus exists that variation in plant water losses leaves few room to the cuticle which should remain with high efficiency in most conditions. However, stomatal closure augments the proportion of water which is lost through the cuticle then variation in its composition or thickness may have amplified influence on leaf transpiration in such conditions. Recently, nighttime transpiration in *Zea mays* was shown to be genetically controlled by putative genes involved in cuticular biosynthesis (Lin et al., 2020). An advantage of breeding on the cuticle for high WUE is that it does not directly interfere with stomatal behaviour and photosynthesis. However, the factors determining cuticular permeability as well as the control of the cuticle on transpiration are not well-understood.

Towards more water-use efficient grapevine varieties

Viticulture covers a large part of arable lands in the Mediterranean region (e.g. 27 % in Languedoc Roussillon). It is one of the rare crops suited to these areas where rainfall is rare in summer, soil water retention capacity is limited and soils may be tricky to till. For these reasons and other, historical ones, there are few alternatives in such regions to viticulture which plays multiple roles in economy, prevention of hydrological risks and tourism. However, between 25 to 73% decrease in major wine producing regions could become unsuitable for this production in 2050 due to higher frequency of drought and heat stress episodes with climate change (Hannah et al, 2013). Several research projects therefore explore innovative avenues based on crop management and/or scion and rootstock selections to mitigate the negative impacts of warm and dry conditions (van Leeuwen et al., 2013). A potentially powerful mean is the selection and breeding of plant material with improved WUE (Bacon, 2004; Condon et al., 2004; Duchêne, 2016). The grapevine germplasm possesses huge variation to alleviate the climate change losses (Morales-Castilla et al., 2020). Recently, genetic variation for nighttime transpiration was detected and revealed that genotypes with reduced nighttime transpiration were not systematically impaired in growth and displayed increased WUE (Coupel-Ledru et al., 2016). This paves the way for the breeding of water-use efficient varieties by reducing nighttime water losses.

Control of grapevine transpiration

Although the importance of controlling water losses for grapevine, little is known about the molecular and physiological actors controlling transpiration in this species. Genomic regions, so-called quantitative trait loci (QTLs), within grapevine have been identified controlling transpiration in well-watered, dry or both conditions (Coupel-Ledru et al., 2014; Marguerit et al., 2012). Both the scion and the rootstock may genetically control transpiration (Coupel-Ledru et al., 2014; Marguerit et al., 2012).

The rootstock may affect stomatal opening via hormonal and hydraulic signalling from the root to the shoot via ABA production (Marguerit et al., 2012) or aquaporins (Perrone et al., 2012; Pou et al., 2013).

The scion is thought to play a major role in controlling leaf transpiration as it regulates stomatal development and originates many signals affecting stomatal aperture. Previous results observed a positive relationship between stomatal density and nighttime transpiration in grapevine (Coupel-Ledru et al., 2016). The genes controlling the stomatal development are well-known in the model plant *Arabidopsis thaliana* (Bergmann and Sack, 2007) and shared across species (Chater et al., 2017). Accordingly, homologs of stomatal development genes may be identified using the grapevine genome assembly (Jaillon et al., 2007) and annotation (Canaguier et al., 2017). There exists great genotypic variability within grapevine for the number of stomata (Gago et al., 2019; Teixeira et al., 2018), but so far the molecular actors controlling variation in stomatal density in grapevine have not yet been identified.

A second factor contributing to nighttime transpiration may be incomplete stomatal closure in response to darkness (Coupel-Ledru et al., 2016). Several homologs of genes known to be involved in stomatal opening or closure have shown to be active in the grapevine stomatal guard cells: the R2R3-MYB transcription factor *vvMYB60* (Galbiati et al., 2011) and a KAT-type channel called *SIRK* (Pratelli et al., 2002). Another grapevine transcription factor, *vvNAC17*, may also be involved in the control of stomatal opening and density in response to ABA (Ju et al., 2020). Wild-growing grapevine species *Vitis amurensis* Ruprecht (Asian group) is known to be tolerant to abiotic stresses for which the Ca^{2+} -reactive CDPKs may be responsible (Dubrovina et al., 2013, 2017), potentially regulating guard cell anion channels as *SLAC1* (Geiger et al., 2010). Moreover, sugar signalling by trehalose or trehalose-6-phosphate has been found to alleviate the effect of ABA to close stomata in epidermal peels of grapevine (Gamm et al., 2015). Interesting insights on the control of stomatal aperture in grapevine arises from research on plant pathogens that enter the leaf via open stomata. Grapevine downy mildew (*Plasmopara viticola*) is an oomycete that opens stomata in order to enter the leaf and create infection thereby creating a higher vulnerability to water starvation (Allègre et al., 2007). The stomatal closure response upon infection is linked, as for ABA, to ROS production (Allègre et al., 2009). Also lipid metabolism may be involved via the phospholipase *vvPLA₂* (Laureano et al., 2018), known to be involved in light-induced stomatal opening (Seo et al., 2008). Finally, upon infection with pathogenic bacteria and downy mildew, actin filament reorganization was observed in the guard cells and spreading to adjacent epidermal cells, forming the defence reaction (Guan et al., 2014). Actin reorganization was not directly linked with stomatal closure but may be important for regulation of signal transduction by regulation of ion channels (Hwang et al., 1997).

Variation in the level of physiological actors as trichomes or cuticle has been described in grapevine but is not yet linked to its impact on leaf water losses. The cuticular composition and structure has been mainly investigated on grape berry during ripening (Arand et al., 2021; Dimopoulos et al., 2020), also linked to berry disease appearance (Commenil et al., 1997). The cuticular organisation on the adaxial side of leaves was changed upon drought stress (Salem-Fnayou et al., 2011). Differences in leaf cuticular structures have been observed between *Vitis* species (Ickert-Bond et al., 2018) and within *Vitis vinifera* (Monteiro et al., 2013; Teixeira et al., 2018). Trichomes are used to classify grapevine genotypes as they show great genotypic variability which is stable across environments. The density of trichomes is linked to disease resistance (Kono et al., 2018) or presence of predatory mites (Barba et al., 2019). The contribution of leaf micromorphology on transpiration remains unclear.

Outline of the PhD manuscript

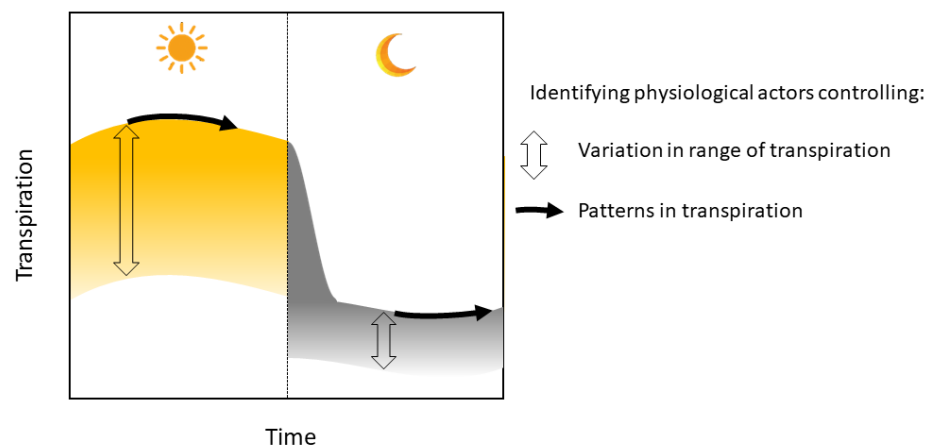


Figure 4: Graphical representation of the aim of this thesis. The main goal is to elucidate the physiological actors underlying genetic variation for transpiration level and patterns at day and at night in grapevine.

The physiological processes by which genomic variation of the scion controls day and night transpiration are unknown in *Vitis vinifera* L.. Identification of the underlying mechanisms is required to avoid unwanted trade-offs on growth when breeding for water-use efficient varieties with low nocturnal water losses. Therefore, this PhD aims to understand the mechanisms by which genomic regions control day- and nighttime transpiration (Figure 4).

We hypothesize that genetic variation for nighttime transpiration is mainly associated with endogenous patterns in stomatal movement regulated at the leaf scale. A high-throughput phenotyping pipeline was designed that allowed to follow daily stomatal patterns. In Chapter 2, I explain the design, analysis and application of this protocol to plants of *Arabidopsis thaliana* and grapevine. Then, it is investigated if the protocol can be applied to excised grapevine leaves to detect genetic variation for patterns in stomatal movement. This was done using a recently created association panel representing the genetic diversity within *Vitis vinifera* L. (Nicolas et al., 2016). Linkage disequilibrium is low in this population, permitting the analysis of candidate genes.

Making use of the developed protocol, the third objective is to run a GWAS on grapevine and look at candidate genes to identify some of the physiological processes controlling daytime transpiration in grapevine. In Chapter 3, GWAS performed on average daytime transpiration level in grapevine enabled us to identify *MEX1* as a candidate gene. Phenotyping the *Arabidopsis mex1* mutant revealed its impairment in light-induced stomatal opening and endogenous nighttime reopening. Screening of a collection of *Arabidopsis* mutants impaired in starch turnover stressed the role of *MEX1* in stomatal responses to environmental changes. The model plant *Arabidopsis* allowed to dissect the signals between mesophyll, guard cells and their impact on diel patterns in stomatal opening.

In Chapter 4, the objective is to identify the physiological determinant underlying the most significant genomic region controlling nighttime transpiration of detached leaves from the association panel. Investigation of the function and expression of the genes in the region around the marker is done to identify candidate genes and processes. Then, genetic analysis for potential candidate processes is performed after phenotyping stomatal density, trichomes and cuticular ridges around stomata. This is combined with physiological assays using excised leaves of contrasting genotypes to understand the mechanisms by which this region controls nighttime transpiration.

In Chapter 5, we discuss the obtained results in this PhD in the perspective of breeding for high WUE in grapevine.

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Chapter 2

A high-throughput phenotyping pipeline to detect genetic variation for diel transpiration patterns

Outline

In this chapter I describe the development of a high-throughput protocol to monitor diel transpiration rates in controlled conditions. Close monitoring of transpiration rate is needed to analyse stomatal opening or closure patterns during the day and night. The advantages and challenges of the protocol and analysis pipeline are shown by using examples of transpiration patterns recorded with *Arabidopsis thaliana* plants. The protocol is then applied to grapevine whole-plants, with scales placed in controlled or outdoors conditions, and to detached leaves in an automaton. The power of the method is illustrated on detached grapevine leaves for the detection of genetic variation in transpiration patterns.

Contributors

This chapter was based on research I conceptualized with my supervisors Florent Pantin¹ and Thierry Simonneau¹. For data collection, I was helped by Lucille Roux¹ (grapevine GWAS experiment), the postdoctoral researcher Aude Coupel-Ledru¹ (grapevine GWAS experiment) and Myriam Dauzat¹ (PHENOPSIS monitoring). I have performed all data analyses, after initial advices on grapevine quantitative genetics by Aude Coupel-Ledru¹, Timothée Flutre² and Agnès Doligez³. I wrote the chapter with the help of Florent Pantin¹ and Thierry Simonneau¹.

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Abstract

Plant transpiration is mainly caused by stomatal pore opening to allow atmospheric CO₂ entry for photosynthesis. Generally, stomata open in the morning in response to light, reopen slowly until midday and close gradually in the afternoon. At night, stomata close strongly, but tend to reopen during the night. These diurnal and nocturnal patterns are observed in both natural and controlled conditions, suggesting that endogenous processes control stomatal behaviour during the diel cycle (24h). Both patterns may augment 'inefficient' water losses as the coordination with carbon gains is absent, thereby decreasing the water-use efficiency. Here, we developed a protocol to monitor diel transpiration patterns in *Arabidopsis thaliana* and grapevine based on high-throughput gravimetry in controlled conditions. Furthermore, a data processing pipeline is developed to clean gravimetric data before estimation of transpiration. The protocol is applied to detached grapevine leaves in artificial xylem sap and analysed for the appearance of senescence symptoms. Then transpiration patterns of detached leaf, whole-plant and leaf gas-exchange are compared in either controlled or outdoor conditions. Finally, genetic variation and QTLs was found for a diurnal transpiration pattern using a grapevine association panel consisting out of 279 varieties. These results may help to understand the control of stomatal movements in order to optimize plant water use.

Introduction

The 24-hour (diel) variation in plant water losses is mainly caused by stomatal pore opening to allow atmospheric CO₂ entry for photosynthesis. Stomatal aperture is intimately connected to the diurnal pattern of sunlight intensity: stomata gradually open until midday and slowly close in the afternoon (Matthews et al., 2018, 2019; Rogiers et al., 2009). This typical sinusoidal pattern is also observed when plants are exposed to constant light intensity in laboratory conditions (Gorton et al., 1989; Kaiser and Kappen, 1997; Lanoue et al., 2018; Lebaudy et al., 2008; Matthews et al., 2017; Webb, 2003), suggesting a light-independent control of diurnal stomatal behaviour. At night, stomata close, but tend to reopen in uncontrolled (Bucci et al., 2004; Donovan et al., 2003; Howard and Donovan, 2007, 2010; Resco de Dios et al., 2016; Schwabe, 1952) and controlled conditions (Easlon and Richards, 2009; Hassidim et al., 2017; Resco de Dios et al., 2015). The dynamic behaviour of stomata in stable environmental conditions suggests that stomatal aperture is regulated by endogenous processes. Both diurnal and nocturnal stomatal patterns may increase ‘inefficient’ water losses by the apparent absence of the coordination with carbon gains (Matthews et al., 2017). The coordination between water losses and carbon gains determines the water-use efficiency of the plant and is linked to yield in dry conditions (Condon, 2020). Therefore, identifying the endogenous signals controlling diel stomatal patterns may help to optimize plant water use.

The swelling and deflation of two guard cells is key to open or close the stomatal pore (Lawson and Matthews, 2020). Guard cells swell by the import of ions and water increasing the turgor pressure. Changes in turgor pressure modifies the form of the guard cell from straight to a bended shape. The bending of both guard cells result in the opening of the pore (Woelfenden et al., 2018). Turgor loss by the export of ions and water unbends the guard cells thereby closing the stomatal pore (Kim et al., 2010). The export and import of ions at the guard cell membrane is regulated by a variety of endogenous signals from which the stomata-closing hormone abscisic acid (ABA) is best known (Raghavendra et al., 2010). ABA accumulation is regulated by the circadian clock (Legnaioli et al., 2009; Robertson et al., 2009), which orchestrates a 24-hour biological rhythm found in the majority of eukaryotes to confer adaptive advantages (Caldeira et al., 2014; Dodd et al., 2005; Green et al., 2002). The key clock regulator TOC1 (TIMING TO CAB EXPRESSION 1) modulates ABA signalling and has been hypothesized to change stomatal apertures during the diel cycle (Pokhilko et al., 2013). Alternatively, increased delivery of ABA at the guard cells by the transpiration stream may modify stomatal opening (Tallman, 2004). The circadian clock is important to optimize carbon use for plant growth (Dodd et al., 2005; Graf et al., 2010) and is itself regulated by sugars from photosynthesis (Bläsing et al., 2005; Seki et al., 2017). The strong correlation between photosynthesis and stomatal aperture suggests the existence of a photosynthesis-derived signal influencing stomatal aperture. The nature of this signal has not yet been identified but may comprise the sensing of guard cells to direct changes in intracellular CO₂ concentration (Hedrich and Marten, 1993; Mott, 1988) or indirectly via a photosynthesis-derived product as ATP (Tominaga et al., 2001), malate (Hedrich and Marten, 1993; Hedrich et al., 1994; Lee et al., 2008) or sugars such as glucose and sucrose (Daloso et al., 2016; Flütsch et al., 2020a, 2020b). Interestingly, higher daytime photosynthesis levels increased the nighttime stomatal pattern in *Vicia faba* (Easlon and Richards, 2009). Starch accumulation by photosynthesis could mediate the link between daytime conditions and nighttime stomatal aperture. Accordingly, the starch-deficient mutant *pgm* was impaired in the increase in stomatal conductance during the night compared to the wild-type (Lascève et al., 1997). Starch is accumulated during the day and broken down at night to sustain growth in the absence of photosynthesis (Zeeman et al., 2004). The sugars produced by nighttime starch degradation diffuse from the chloroplast into the cytosol (Zeeman et al., 2004). The accumulation of sugars in the cytosol of guard cells may trigger stomatal opening by providing osmotica or energy. Recently, glucose importers at the plasma membrane of the guard cell

were characterised, increasing stomatal opening in response to light (Flütsch et al., 2020a). Alternatively, the accumulation of CO₂ within the leaf by nighttime respiration may trigger stomata to open, in contrary to the closing response of stomata to daytime increases in CO₂ (Fricke, 2019).

Genetic analysis can be a powerful tool to identify the underlying processes controlling diel stomatal patterns and provide insights in the selective advantages of diel stomatal regulation. This requires the screening of large populations facilitated by the development of high-throughput methods. Gas-exchange measurements on leaves enclosed in cuvettes have revealed that variation exists for diurnal stomatal dynamics between species (Howard and Donovan, 2010; Matthews et al., 2017) and mutants impaired in the circadian clock or stomatal light responses (Dodd et al., 2005; Hassidim et al., 2017; Wang et al., 2020). However, gas-exchange measurement in controlled-environment cuvettes require long equilibration times and are not well suited to high-throughput characterisations. Direct measurement of stomatal apertures can be done microscopic imaging of epidermal peels (Gorton et al., 1989), but is difficult to scale up in controlled conditions and limited to certain species. Thermography has been useful to identify mutants impaired in stomatal regulation (Costa et al., 2015; Merlot et al., 2002), but requires several additional steps including energy balance equations to derive transpiration and stomatal behaviour (Viale-Chabrand and Lawson, 2019, 2020).

Here, we present a high-throughput method to monitor transpiration by gravimetry in controlled conditions. A phenotyping robot for growth of *Arabidopsis thaliana* was adapted to monitor the weight of up to 150 individuals per 30 minutes with a mobile scale (Granier et al., 2006a). Additionally, we developed an automated pipeline to clean weight data and to derive reliable diel transpiration dynamics with a temporal resolution of 30-min. Transpiration dynamics can be used as a proxy for stomatal opening provided that factors such as environmental conditions, growth and leaf movements are carefully monitored. This method was applied on whole-plants of the model species *Arabidopsis thaliana* and on detached leaves of *Vitis vinifera* L., a crop species of high economical interest threatened by climate change (Hannah et al., 2013). The effect of rosette growth on the estimated transpiration pattern was investigated using *Arabidopsis thaliana*. Then two experiments were carried out to test the feasibility of using detached grapevine leaves to monitor stomatal patterns: 1) senescence symptoms were monitored on transpiration of detached leaves during 48 hours and 2) the transpiration patterns of detached leaves, whole plants and gas-exchange were simultaneously measured and compared. Detached leaf assays offer several advantages: it requires little space allowing more replicates and stomatal behaviour can be instantaneously measured on mature, fully expanded leaves with stable area. This confers important advantages over measurements on whole plants using sap flow gauges (O’Keefe et al., 2020) or gravimetry (Cirelli et al., 2012; Weksler et al., 2020) whereby factors as uncontrolled distribution of leaf age, growth of young leaves and leaf movements can mask stomatal behaviour. Transpiration patterns were observed on detached leaves during the day and to a lesser extent at night. Similar patterns were observed during the second day and night, showing close similarity to the transpiration pattern of whole-plants and leaf gas-exchange. Finally, our method allowed us to detect genetic variation for a stomatal closure pattern in the afternoon. Together, these results will help to identify the signals originating from endogenous processes that control diel stomatal patterns.

Material and Methods

The objective of this chapter is to develop a high-throughput protocol that allows the detection of genetic variation for patterns in stomatal opening in controlled conditions in the species *Arabidopsis thaliana* and *Vitis vinifera*.

Development of a high-throughput protocol to monitor diel transpiration patterns

A pipeline was developed that estimates transpiration patterns from gravimetric data measured by a scale (Figure 1).

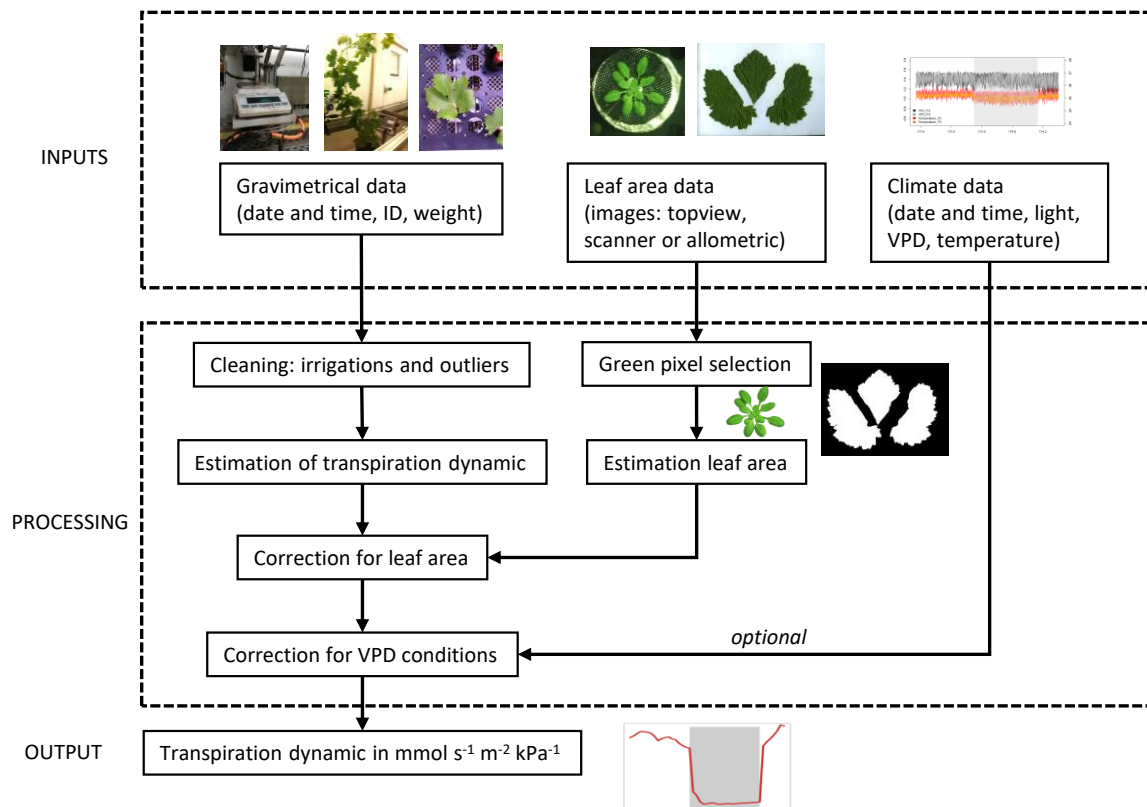


Figure 1: Workflow diagram presenting the steps to calculate transpiration patterns from gravimetric data. Gravimetric data is cleaned for aberrant points and corrected for the surface area. Climate data is used to check stable conditions and to correct for different VPD levels, if necessary.

Gravimetric data

The gravimetric data can come from all types of scales or any calibrated force sensor (Figure 1). When the goal is to estimate transpiration rate at a small temporal scale, it is important that the water lost per time unit falls within the precision of the scale. For example, plants of *Arabidopsis thaliana* had a minimal rate of weight loss of around 0.01 g per hour, so per hour we may use a precision of 0.01 but when interested in transpiration rate every 30 minutes we need a scale with a 0.001 precision. The higher precision, the higher the influence of unstable measurement conditions as turbulence on the measurement.

When using high-precision scales, a mobile scale is preferred instead of a constant load on an immobile scale. Maintaining a constant load on the high-precision scale often results in erroneous measurements caused by a drift. Therefore, the scale was installed on a moving automaton in a climate chamber called PHENOPSIS (Granier et al., 2006a). The scale moves under the pots, and ascends the

plant to measure its weight. The mobile scale is able to measure the weight of maximal five plants per minute, depending of the distance between pots.

High-precision scales require stable surrounding conditions in order to stabilize and to weigh correctly. This is generally not the case in climate chambers as climate control creates turbulent air streams nonstop. Moreover, stabilization is hindered by the constant decreasing weight of the plant by transpiration. To avoid erroneous measurements, stabilization options of the scale were adapted preventing a too long stabilization time prior to each measurement. The weight was only saved when stable, i.e. weight did not change more than 0.01 g during six consecutive points sent by the scale (2400 bits/s). If no stable weight was obtained during 15 seconds, the scale descended, and ascended to try weighing again. This protocol was repeated a maximum of three times to obtain a correct weight.

Cleaning gravimetric data

The gravimetric data was cleaned for irrigation events and outliers (Figures 1, 2). During the experiment, plants or leaves were irrigated to assure well-watered conditions. By using R (R Core Team, 2019), a script was developed to automatically detect irrigation events based on the positive difference between consecutive weights. When weight was recorded continuously by an immobile scale, intermediate points during irrigation were automatically detected and eliminated before the calculation of the added amount of water with a linear regression model. Then, the added water amount by irrigation was subtracted from the weight data after the irrigation event.

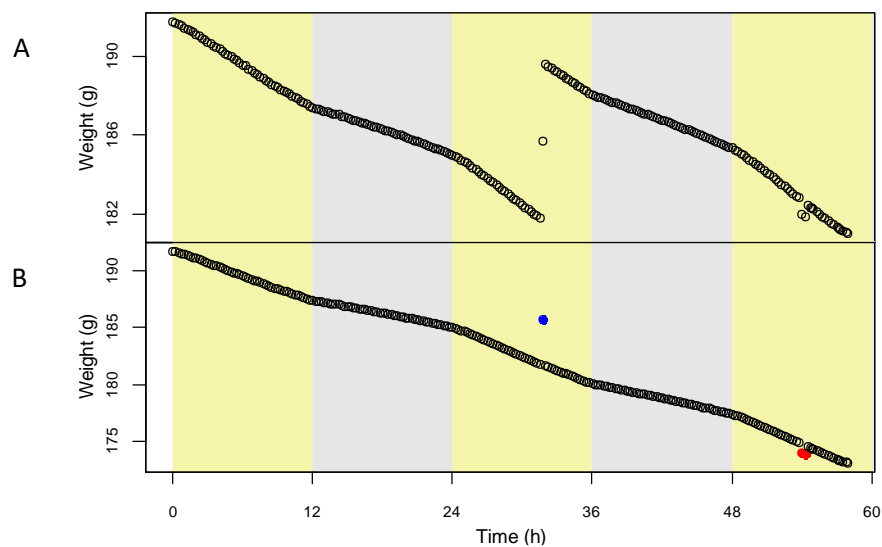


Figure 2: Example of gravimetric output data and cleaning. A) Hypothetical example of the raw gravimetric data during 60 hours including an irrigation during the daytime and outliers, B) the data is corrected for water added by irrigation and outliers (see procedure in main text). The script automatically detects intermediate weights during irrigation (blue point) and outliers with an unexplained nature (red points).

The method to estimate transpiration is highly sensitive to outliers i.e. measurements that violate the assumptions of normal distributed residuals. Outliers may arise from irrigation events or unstable conditions in the climate chamber such as turbulent air streams or contact with neighbouring plant. Also, unintentional variation due to electro-magnetic perturbation was sometimes observed in the

climate chamber. These effects perturb the measurement accuracy and the estimation of transpiration. Therefore, considerable effort was invested in the automatic identification of outliers. A script was developed consisting out of three steps and a final visual inspection of the automatic results. First so-called ‘outlier hotspots’ were identified by comparing the difference between two consecutive points. If the difference was larger than the user-defined biological lower and upper limit for transpiration, the spot was characterised as a ‘outlier hotspot’. The biological limits were defined specifically for the day and night period. Biological limits were not too strict and aimed to identify obvious data spikes (Figure 2, blue point). The second step targeted ‘outlier hotspots’ containing less obvious outliers (Figure 2, red points). These hotspots were detected by using the 1.5 interquartile ranges (IQRs), a classical method to detect outliers (Vinutha et al., 2018). IQRs were calculated using all differences between consecutive points of a light or dark period and hotspots were identified when a point was out of the IQR range. The third step analysed in more depth each outlier hotspot to decide about in- and outliers. Weights in the hotspot were subjected to regression analysis and computing leave-one-out deletion diagnostics to measure the influence of data points. Outliers within a hotspot were identified by using DFFITS (Welsch and Kuh, 1977) which estimates the change of fitted values when deleting each point. A data point was considered to be an outlier when the absolute value of DFFITS was larger than:

$$tolerance * \frac{\sqrt{(p + 1)}}{n - p - 1}$$

here *tolerance* is a number depending on proximity to a day/night transition (when the derivative of weight loss is expected to vary suddenly), *n* the number of observations (weights) and *p* the number of parameters in the regression. Generally, *tolerance* was assigned a value of 2, but when close to a day/night transition this number was increased to allow flexibility. The number of parameters was equal to 2 as the intercept was included.

The hotspot strategy saves considerable computation time and is less-energy-intensive as the regression diagnostics are only calculated for spots possibly containing outliers. However, in the case of consecutive aberrant points, the DFFITS method loses detection power. Therefore, a manual check of every weight loss dynamic was done at the end of this procedure to identify a few remaining false-positives and false-negatives.

Estimation of transpiration

Next, the transpiration rate was estimated based on the cleaned gravimetric data (Figures 1, 3). The slope between two consecutive points represents the rate of water loss, however is sensible to small variations induced by unstable conditions during measurement (black line, Figure 3A). Therefore, a floating linear regression model was used to estimate transpiration rates (red line, Figure 3A). The principle of a floating linear regression model is shown in Figure 3B: every *x* minutes a local linear regression is modelled. In the example we can observe the changes in the rate of water loss during a night to day transition. In PHENOPSIS, weights were recorded at least once every 30 minutes and a 180-min floating time interval selected six data points within the specific day/night period to perform linear regression. The interval size for the linear model can be adapted according to the number of points and rate of weight loss. Although water loss is not a linear process, a local linear model was preferred over additive modelling methods such as LOESS or splines that use a smoothing function over large time periods (Zuur et al., 2009). This was because these methods do not well predict the transpiration rate at the edges of a day/night period or results in unnatural bends in the estimated transpiration curve over time. The floating linear model on his side induces uncertainty at the edges of a day/night period as linear regression was carried out on a smaller number of data points.

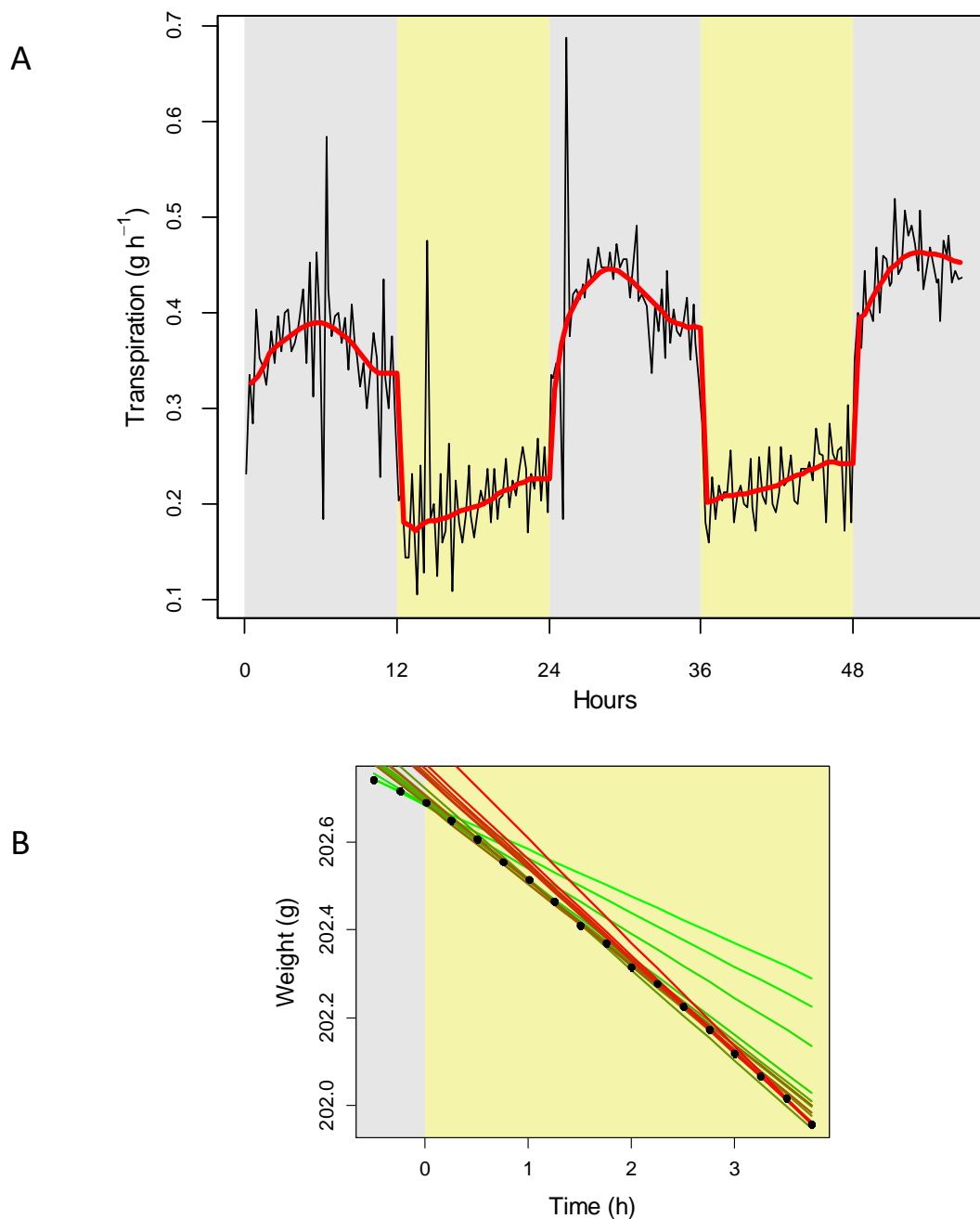


Figure 3: Application and principle of the floating linear regression. A) Transpiration dynamic calculated by two methods: slope between two consecutive points (black) and by floating linear regression (red). The floating linear regression model is less sensitive for outliers and smoothed the data using a floating time interval of 180-min. B) The concept of a floating linear regression is visualized on gravimetric data. The linear model selects points according to a floating time interval and calculates every 15 minutes the slope of the regression line. In this example, we can clearly see the increasing slope with time using a 60-min floating time interval from the first hours after the transition from night (grey area) to day (white area).

Additionally, a bias in the estimation of transpiration rate is created before a day/night transition as the interval for the regression contained more and more the same set of data points. This resulted in

the typical flattening of the transpiration rate curve before a transition (red line, Figure 3A). Choosing the floating linear regression method also permitted us to adapt the floating time interval according to the proximity of a transition. For example, floating time interval at the start of the day or night was shorter than in the middle of a period, enabling the detection of sudden changes in transpiration rate as observed upon opening and closing of stomata in response to light or darkness.

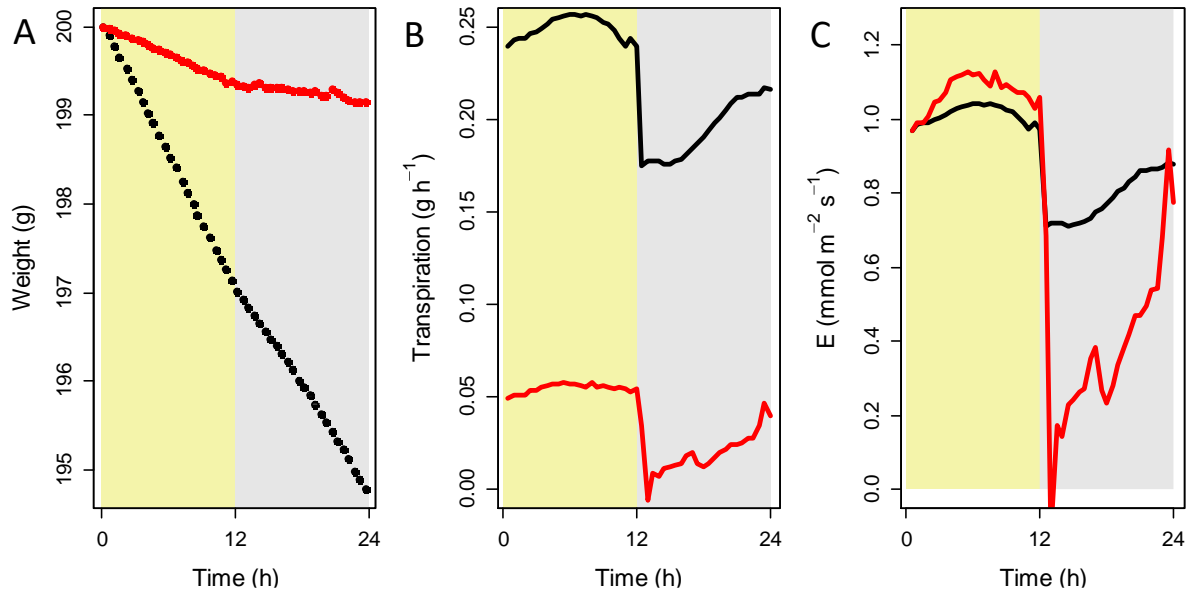


Figure 4: Transpiration speed influences the accuracy of the estimation of transpiration by floating linear regression. Fast (black) and slow (red) transpiring *Arabidopsis* plant. Low water loss speed results in a lower accuracy of the estimation of the transpiration. A) The gravimetric data of both plants during day and night (grey). B) The transpiration dynamic estimated by a floating linear model on both plants. C) The transpiration dynamic corrected for a fixed plant surface.

The choice of a floating time interval that fits all weight loss dynamics without over-smoothing transitions in transpiration regimes is a tricky problem. This floating time interval is a way to reduce uncertainty and avoid spikes in the calculated rate of transpiration as in Figure 3A. However, the relative importance of spikes is also dependent on the mean rate of water loss. Unstable environments can increase the noise on the measurement, resulting in a higher variability of weight recordings. Consequently, at low levels of water losses, relative error on calculated rate of transpiration is higher. This is shown in Figure 4, where the dynamic of weight is represented by the red line for a small *Arabidopsis* plant (~400 mm²) and by a black line for a large plant (~4000 mm²). Especially low nighttime water losses (0.01 – 0.04 g h⁻¹) resulted in less accurate estimation of the slope (Figure 4A). This higher variability is illustrated in the calculation of specific transpiration rate at night (Figure 4B), normalised for the rosette area considered here as stable during the time period (Figure 4C). A solution could be to adapt the floating time interval to the rate of water loss. However, this solution was not preferred as the size of the floating time interval influences the dynamicity of the transpiration rate by smoothing more or less the patterns in transpiration rate. To avoid misleading conclusions on the dynamicity of a certain genotypes, we fixed the floating time interval and used at least five repetitions to obtain a representative transpiration dynamic (Figure 5).

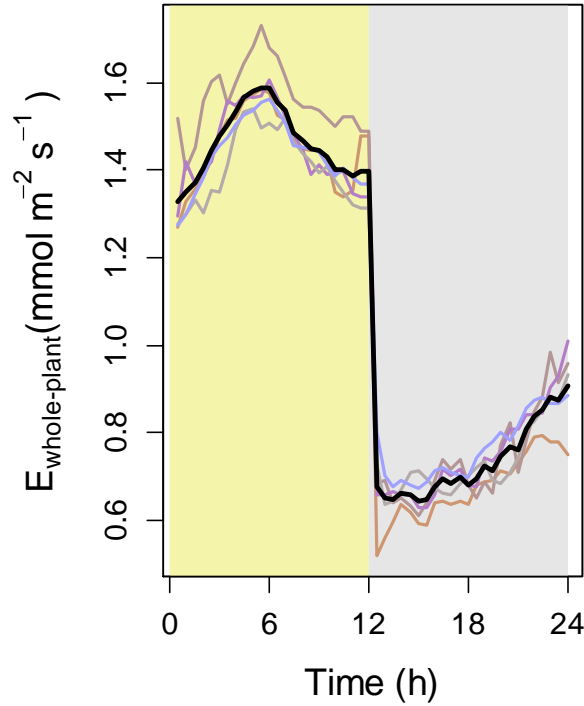


Figure 5: Repetitions are needed to obtain reliable transpiration dynamics. Five Col-0 Arabidopsis plants were regularly weighted in the PHENOPSIS automaton and plant transpiration rate was estimated by using floating linear regression for weight over time after correction for outliers as described in the text. The black line represents the average of five plants.

Correction for leaf area

The estimated transpiration is afterwards corrected for the leaf area in order to compare leaf conductance per unit of evaporative surface (Figures 1, 4B, 4C). The leaf area should be estimated at a frequency depending on the growth speed of the plant. If growth is assumed to be linear during the period, the surface can be calculated by a linear model and interpolation.

Correction for VPD conditions

Plant transpiration is highly dependent on climatic conditions such as light (PAR), humidity, wind speed, temperature, VPD and CO₂. Climate chambers offers a control on these conditions and enable transpiration assays to be repeated in comparable conditions. However, depending on regulation capacity in the chamber, the conditions may to some extent depend on outside conditions as humidity, temperature and CO₂. The saturating vapour pressure (SVP, kPa) was calculated by:

$$SVP = \frac{610.78 * 2.7728^{\frac{T}{283.3}}}{1000}$$

where T is the air temperature in Celsius. Then the vapour pressure deficit was calculated by:

$$VPD = SVP * (1 - \frac{RH}{100})$$

where RH represents the relative humidity.

The VPD is a measure of the 'dryness' of the air and the driver of plant water losses from the leaf to the atmosphere. When unstable VPD values occurred during the experiment, transpiration rates were

corrected for the VPD level by dividing specific transpiration rate by the VPD level at the same time to allow better comparison of leaf conductance between experimental days (Figure 1).

Application of the pipeline

Using the above-described protocol, experiments were carried out to detect variation in transpiration patterns in *Arabidopsis thaliana* and *Vitis vinifera*, listed in Table 1.

Experiment	Species	Experimental climate conditions	Plant level	Genotypes	Method	Replicates	Question
1	<i>Arabidopsis thaliana</i>	VPD 1.1, PAR 180, constant temperature 21, 12/12 hours day/night	whole-plant	Col-0	gravimetric	6	Effect of rosette expansion on transpiration patterns?
2	<i>Vitis vinifera</i>	VPD 1.5, PAR 180, constant temperature 25, 14/10 hours day/night	Detached leaves	Subset F1 population Syrah x Grenache (70 genotypes)	gravimetric	70	After how much time does senescence affects transpiration in detached leaves?
3	<i>Vitis vinifera</i>	VPD 1.1, PAR 180, constant temperature 25	Whole-plants, detached leaves, attached leaves	Association panel	gravimetric, gas-exchange	6	How does transpiration measured by different methods and plant levels relate?
5	<i>Vitis vinifera</i>	VPD 1.5, PAR 180, constant temperature 25, 14/10 hours day/night	Detached leaves	Association panel (279 genotypes)	gravimetric	6	Genetic variation for patterns in stomatal opening?
4	<i>Vitis vinifera</i>	outdoors (July 2020, Montpellier), indoors: VPD 1.5, PAR 180, constant temperature 25, 14/10 hours day/night	Whole-plant, detached leaves	Syrah	gravimetric	6	What are the differences between outdoor and indoor transpiration?

Table 1: Overview of the five experiments presented in this chapter using our pipeline. The first experiment tests the effect of rosette expansion on estimated transpiration patterns in *Arabidopsis thaliana*. Then our developed protocol was applied on detached leaves of *Vitis vinifera* to test its feasibility to follow transpiration patterns during day and night. Next, transpiration patterns of whole vines (in and outdoors), leaf gas-exchange and detached leaves are compared. Finally, an association panel consisting out of 279 grapevine varieties is used to detect genetic variation for patterns in stomatal opening. Per experiment the experimental climatic conditions (VPD in kPa, air temperature in °C and light in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), plant level, genotype, number of replicates and question are shown.

Arabidopsis thaliana

The plants were grown in the PHENOPSIS platform (Granier et al., 2006b). *Arabidopsis* seeds of Col-0 were sown in 200-ml cylindrical plastic pots filled with a weighed amount of a soil mixture containing 30:70 (v/v) loamy soil and organic compost. The experiment was carried out on 28-days old plants. Plants were grown under 12-h photoperiod at an irradiance of $180 \mu\text{mol m}^{-2} \text{s}^{-1}$, with constant air temperature (22 °C) and relative humidity (60%) during day and night. Each pot was weighed daily and its soil water content kept constant ($1.4 \text{ g water g}^{-1} \text{ dry soil}$) by watering with a modified Hoagland solution.

At the start of the experiment, soil evaporation was avoided by covering the soil with a plastic film. The weight was automatically monitored every 30 minutes during day and night by a high-precision scale (0.001, Precisa XB 620M, Precisa Instruments AG, Dietikon, Switzerland). Irrigation was performed manually in order to maintain the target soil water content.

The estimation of the transpiration dynamic was done by a floating linear regression after cleaning of the gravimetric data. Then, the transpiration dynamic was corrected for the surface, estimated by a linear model using the projected leaf area. The projected leaf area of *Arabidopsis thaliana* was derived from top view RGB images captured daily by the automaton in PHENOPSIS. Pixels corresponding to leaf area were segmented from the background by three threshold algorithms using Python (van Rossum and Drake, 2003). First, an RGB threshold selected green pixels. Then, a threshold was applied based on the ratio of the green channel to the red and blue channel. Afterwards a HSV threshold was applied with help of the matplotlib library (Hunter, 2007). Finally, leaf or rosette contours were drawn with OpenCV libraries (Bradski, 2000) and a final mask was created by including all the pixels within the contour higher than a minimum pixel value. Projected leaf area strongly correlates with total leaf area as leaves are horizontally orientated towards the camera and leaves are avoiding shading each other (Arend et al., 2016).

Vitis vinifera

Experiment 2

The second experiment (Table 1, experiment 2) was performed on detached grapevine leaves of 68 randomly-chosen genotypes of the mapping population between Syrah × Grenache (Adam-Blondon et al., 2004; Huang et al., 2012). Two-years old plants in 3L pots derived from cuttings grafted on the rootstock 110R were grown outside in the Ecotron located in the Pierre Galet experimental vineyard (INRAE-SupAgro campus, Montpellier, France). Vines were pruned to produce a single shoot and cut at a height of 3 meters. Two-weeks before the start of the experiment, plants were transferred to a greenhouse exposed to a relative humidity of 50% and a temperature of 20-30°C on average. On sunny days the average PAR was $550 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and on cloudy days $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The experiments were carried out in July 2018.

At the start of the experiment, mature grapevine leaves which were fully expanded were cut at the base of the petiole, immersed in distilled water and recut to avoid air occlusion and subsequent cavitation. These leaves were generally situated between the 10th and 20th phytomer from the apex. Leaf movements were avoided by fixing the petiole at the level of the pulvinus on the tube by adhesive putty. Leaves were then assigned to solutions in 50 mL tubes filled with degassed artificial xylem sap (AXS) containing $2 \text{ mol m}^{-3} \text{KH}_2\text{PO}_4$, $1 \text{ mol m}^{-3} \text{MES}$ and $0.4 \text{ mol m}^{-3} \text{Ca}(\text{NO}_3)_2$ (Coupel-Ledru et al., 2016), with their petioles tightly sealed to the containers caps to prevent direct evaporation of AXS. Generally, leaves were installed in the morning around eleven o'clock local time in PHENOPSIS and exposed to stable day and nighttime conditions for VPD (1.5 kPa), air temperature (25 °C) and light ($180 \mu\text{mol}$

photons $\text{m}^{-2} \text{s}^{-1}$). The lights were shut off at the same time as dusk in natural outdoor conditions and turned on at natural dawn (10-hours).

The estimation of the transpiration dynamic was done by a floating linear regression after cleaning of the gravimetric data. Then, the transpiration dynamic was corrected for a stable surface using leaf scans. Detached grapevine leaves were scanned after transpiration assay using a flatbed scanner (Epson 4990, Seiko Epson Corp., Nagano, Japan). Afterwards the total leaf surface was calculated the Python script as used for *Arabidopsis* (described above).

Experiment 3

In the third experiment (Table 1, experiment 3), the detached leaf transpiration was compared to whole-vine transpiration and leaf gas-exchange. Two-years old plants in 3L pots of four genotypes were used (Mondeuse Blanche, Salicette, Mskhali and Gantziandan). Detached leaves were harvested at the end of the afternoon, installed in PHENOPSIS and monitored following the protocol described above (only not degassed). At the same time, two pots per genotype were well-watered, bagged to avoid evapotranspiration of the soil and installed in PHENOPSIS on an immobile scale with a precision of 1 g (OHAUS Defender 5000, Parsippany, NJ, USA). The scale was connected to a data logger and the weight was recorded every 15 minutes. The transpiration of one whole-vine leaf was placed in a leaf cuvette (4 cm^2) of a Li-Cor 6400 gas analyser system (Li-Cor Inc., Lincoln, NE, USA) connected to a dew-point generator to control humidity (Li-Cor 610). The whole-plants, detached leaves and leaf in the cuvette were exposed to stable day and nighttime conditions for VPD (1.5 kPa), air temperature (25°C) and light ($180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The lights were shut off at the same time as dusk in natural outdoor conditions and turned on at natural dawn (10-hour night). At the same day, the experiment was repeated in another PHENOPSIS with increased levels of CO_2 (800 ppm). The estimation of the transpiration dynamic was done by a floating linear regression after cleaning of the gravimetric data. The transpiration dynamic was corrected for the surface by either the scanning the grapevine leaf (described above) or using an allometric relationship between secondary vein length and leaf area (for whole-vine surface at the end of the experiment). This was shown to be a good predictor of leaf area in grapevine (Guisard et al., 2010).

Experiment 4

The fourth experiment (Table 1, experiment 4) aims to detect genetic variation for stomatal patterns using 279 cultivars of a grapevine diversity panel (Nicolas et al., 2016). Plants were generated from cuttings obtained from the Vassal-Montpellier Grapevine Biological Resource Center (Marseillan-Plage, France) in January 2018. The shoots were cut in pieces containing two buds from which the lower one was removed to promote root growth. Around 22 cuttings per genotype were rehydrated in water overnight and planted in rock wool with a root-promoting hormone (auxin, indole-3-acetic acid). The rock wool trays were constantly subirrigated by water heated to a temperature of 24°C . The greenhouse conditions were adapted to favour root growth by minimising direct sunlight radiation by a cover and cooler temperatures $10\text{--}25^\circ\text{C}$. After two-months, ten vigorous cuttings per genotype were transplanted in individual 3L pots with a mixture containing 30:70 (v/v) loamy soil and organic compost. After an initial acclimatization period in the greenhouse, plants were transferred in June to the nursery at the Pierre Galet experimental vineyard of INRAE-SupAgro (Montpellier, France). Ten plants per cultivar were pruned to produce a single shoot, cut at a height of 3 meters and ferti-irrigated to a well-watered regime. Young flowers were systematically removed to avoid differences in vegetative and reproductive stages between genotypes. At the start of the experiment, seventy detached leaves were harvested every morning, installed in PHENOPSIS and monitored following the protocol described above (AXS not degassed). After installation in PHENOPSIS, the leaves are exposed

to stable day and nighttime conditions for VPD (1.5 kPa), air temperature (25 °C) and light (180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The lights were shut off at the same time as dusk in natural outdoor conditions and turned on at natural dawn (10-hours).

The estimation of the transpiration dynamic was done by a floating linear regression after cleaning of the gravimetric data. The transpiration dynamic was corrected for the surface by scanning the grapevine leaf (described in experiment 2), and VPD conditions in PHENOPSIS. Then, a percentage of the decrease in transpiration during the afternoon was calculated as follows:

$$\text{percentage of stomatal closure} = \frac{(E_{\text{midday}} - E_{\text{day-night transition}})}{E_{\text{midday}}} \times 100$$

The data was corrected for spatial variation in PHENOPSIS using kriging (example in Supplementary Figure 2) and Best Linear Unbiased Predictions (BLUPS) were estimated by a linear mixed model with Genotype as random effect (Bates et al., 2015). Next, a genome-wide association study (GWAS) was conducted to identify associations between BLUPS of the association panel and 63,037 SNPs (with minor allele frequency, MAF < 0.05). SNP data were based on microarray and genotyping-by-sequencing (GBS) data followed by imputation (Flutre et al., 2020). The level of relatedness (kinship) between individuals was estimated based on SNPs with a linkage disequilibrium (LD) threshold of 0.5 using the kinship estimator of VanRaden (Flutre et al., 2020; VanRaden, 2008). The weak population structure as described in Nicolas *et al.* (Nicolas et al., 2016) was ignored. GWAS was performed by a multi-locus mixed-model approach (mlmm) (Segura et al., 2012). This method is based on a SNP-by-SNP model implemented in GEMMA version 0.97 (Zhou and Stephens, 2012) and uses a forward-backward stepwise approach to include significant effects in the mixed model, while re-estimating the variance components of the model at each step. Relatedness was controlled by a random effect u , based on additive genetic relationships in a covariance matrix. The model was running on the whole-population of 279 genotypes and maximal number of forward steps was set to 20. At every step, the most significant SNP is included in the model.

Experiment 5

In the fifth experiment (Table 1, experiment 5), the transpiration dynamic of detached leaves was compared with whole-vine transpiration outdoors in the same week in June 2020. For whole-vine transpiration, two-years old Syrah plants (clone 747) grafted on rootstock 110R were transplanted in 9L pots in winter 2019 with a soil mixture containing 30:70 (v/v) loamy soil and organic compost. Vines were pruned to produce a single shoot, cut at a height of 3 meters and ferti-irrigated to a well-watered regime by drip irrigation. The weight of potted grapevines in the natural conditions was monitored using a force sensor with a strain gauge (Micro Load Cell model CZL635, maximum weight 20 kg). Five plants were well-watered and positioned on the sensors while bagging the pot in a plastic bag to avoid evapotranspiration of the soil. Plants were irrigated automatically twice a day during the experiment. The weight is recorded every 30 seconds and saved using a data selector (Campbell Scientific, Leicestershire, United Kingdom). The detached leaves were collected from 20-years old vines in the Pierre Galet experimental vineyard located at the INRAE-SupAgro campus (Montpellier, France). Varieties were grafted on the rootstock Selection Oppenheim No. 4 (SO4) and grown in an artificial soil of sand and small stones completely depending on ferti-irrigation. Six detached leaves were harvested in the morning, installed in PHENOPSIS and monitored following the protocol described above (AXS not degassed). After installation, leaves were exposed to stable day and nighttime conditions for VPD (1.5 kPa), air temperature (25 °C) and light (180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The lights were shut off at the same time as dusk in natural outdoor conditions and turned on at natural dawn (10-hours). The estimation of the transpiration dynamic was done by a floating linear regression after cleaning of the

gravimetric data. The transpiration dynamic was corrected for the surface by scanning the grapevine leaf (described in experiment 2) or using an allometric relationship between secondary vein length and leaf area (for whole-vine surface outdoors). Finally, the transpiration dynamic was corrected for VPD in PHENOPSIS or outdoors (from weather station in the vineyard).

Results

The effect of growth on transpiration patterns in *Arabidopsis thaliana*

The developed protocol was applied to *Arabidopsis thaliana* to investigate how growth may influence the estimated transpiration patterns.



Figure 6: Image of the experimental set up to analyse transpiration patterns in *Arabidopsis thaliana* using PHENOPSIS. Evaporation other than via the plant is avoided by putting a plastic cover on top of the soil. The black plastic grid around the rosette facilitates image analysis to obtain rosette area.

Arabidopsis thaliana is a small, fast growing plant and a model species in plant physiology. The availability of mutants allows to test the effect of a mutation on the plant phenotype. Till now, no high-throughput protocol exists to monitor the stomatal responses to light regimes and daily patterns in stomatal opening. Our developed protocol was applied on whole plants of *Arabidopsis thaliana* in PHENOPSIS (Figures 5, 6). Plant growth may influence the pattern of transpiration by increasing the evaporative area and thus transpiration. Ideally, we would need to know the exact rosette surface at every time when transpiration rate was estimated. However, the automaton cannot image the plants and monitor weight loss simultaneously. Moreover, top view images are not precise enough to follow growth at a small time scale. Therefore, images were generally taken once a day and linearly interpolated assuming constant growth (Figure 7B). However, growth follows a nonlinear 24-hour rhythm that peaks at dawn, decreases during the day and increases gradually at night (Wiese et al., 2007). This may affect the transpiration pattern and leading to misleading interpretations. To investigate the potential effect of growth on the transpiration pattern, images were taken at times close to day-night transition, to analyse the day- and nighttime growth rates. Afterwards, the effect growth rate on the transpiration pattern was modelled to analyse if the transpiration pattern could be completely explained by the growth.

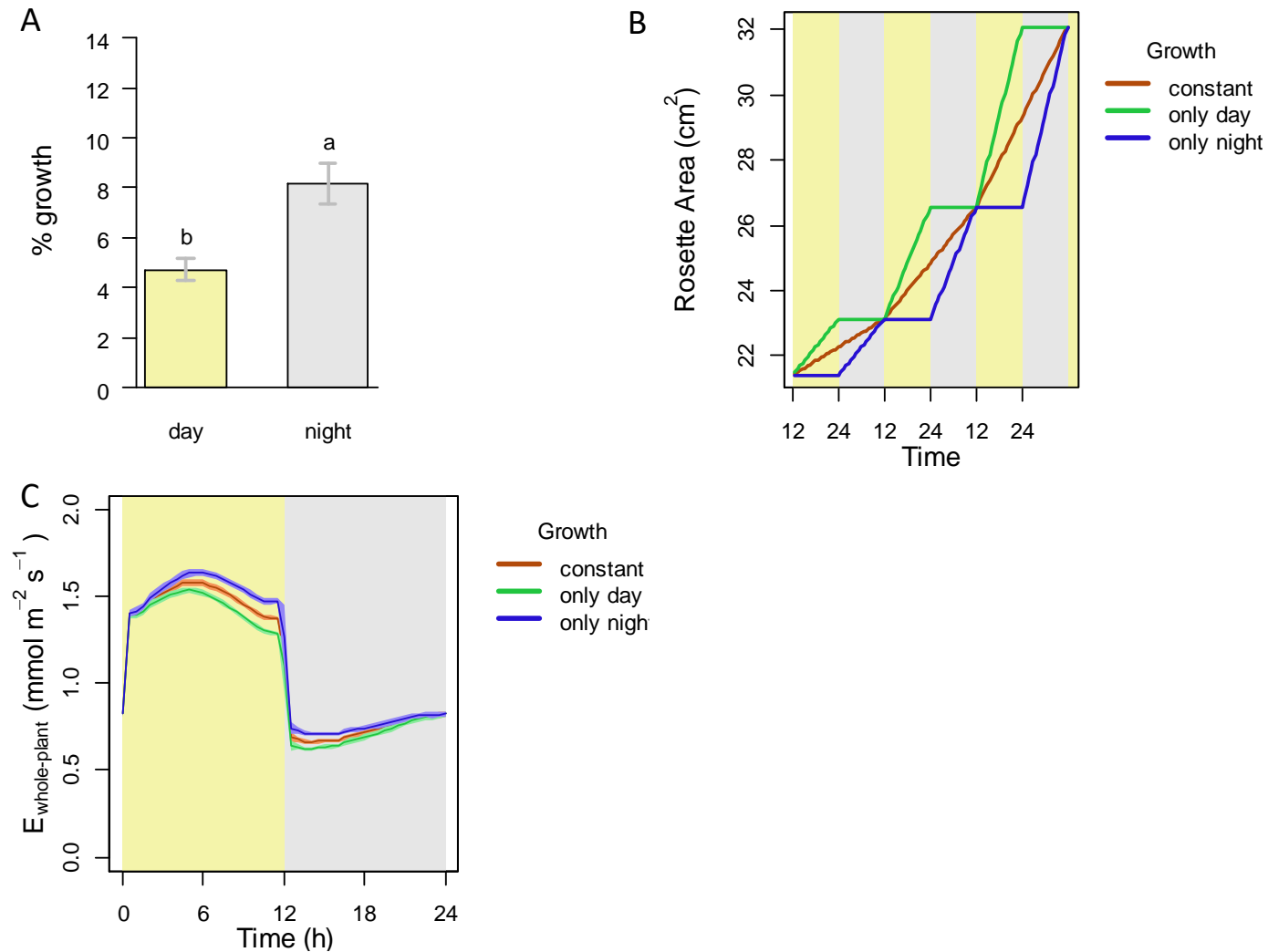


Figure 7: The effect of growth on the specific transpiration dynamic in *Arabidopsis thaliana*. A) Percentage of increase in surface area during day and night of Col-0 plants averaging six consecutive days using five plant replications. Top view pictures were taken at the end of the light period and 30 minutes after the transition from night to day. B) Modelling example of constant growth, only during the day, or only at night for three consecutive days based on the growth of Col-0 plant in PHENOPSIS. C) Effect of constant growth or only during day or night on the estimation of the diel transpiration dynamic. Error bars showing the standard error.

We observed slightly higher growth rates at night than during the day in wild-type Col-0 when comparing images at the start and end of the light periods (Figure 7A). This indicates that the growth rate is different between the day and the night, suggesting the need for a specific surface correction during the day and at night. Since we generally did not have access to such growth dynamics, we investigated the effect of different assumptions for the diel patterns of growth on the estimation of transpiration rates based on actual measurements of plant transpiration rate as described before and of rosette area determined with a 24h time step. We simulated the effect of either constant expansion rate over the 24-h period, or expansion occurring only during the daytime or only during the nighttime (Figure 7B). When expansion was assumed to occur only during daytime, specific transpiration was

logically reduced at the end of the day compared to steady 24-h expansion (Figure 7C) because new leaf area (denominator in the calculation of specific transpiration rate) settled more rapidly. For the same reason, specific transpiration rate decreased in the afternoon more rapidly than it did for plants assumed to grow at constant rate over 24 hours or only at night and remained lower until the last time of calculation when rosette area resumed to the same value for all assumptions. As could be expected, the conclusions were exactly the reverse when assuming that growth occurred later in the nighttime only. Similarly, at night, the different assumptions about the distribution of leaf area expansion were as expected: lower specific transpiration rate was obtained for plants assumed to grow in the first daytime phase only (respectively the last nighttime phase only). More importantly with respect to nighttime dynamics, the different assumptions had the following influences. In the example of Figure 6, transpiration rate slightly increased during the night whatever the assumption made for growth daily pattern. However, still for the same reason, assuming growth to occur only during the first daytime phase (respectively during the ending nighttime phase) lowered (respectively increased) the calculation of specific transpiration rate at the beginning of the subsequent night period). Since growth ended with the same rosette area for all assumed cases, specific transpiration resumed to the same value at the end of the 24-h period. As a result, transpiration rate re-increased more rapidly during the night when growth was assumed to occur early during the daytime phase than later during the nighttime phase. These results indicate that assumption made on daily growth patterns may considerably influence our calculation of specific transpiration patterns in a fast-growing plant as *Arabidopsis thaliana* in laboratory conditions. Nevertheless, even if plants grew only at night, there was still an increase in transpiration observed during the night in our example (Figure 7C) suggesting stomatal opening at night or changes in aerodynamic resistance around leaves. Indeed, leaves bend upwards in response to darkness thereby decreasing the boundary layer resistance for water vapour and increasing transpiration (Hangarter, 1997; Mano et al., 2006). However, strong vertical leaf movements are observed at the start of darkness and gradually decrease during the night as observed from projected leaf area imaging (Flood et al., 2016). So this effect is expected to decrease during the night while we observed an increase in transpiration at night. Calculating the variation at night in transpiration and surface, indicated that growth may explain around 30% of the increase in transpiration at night (Supplementary Figure 1). This strongly suggest that the transpiration pattern at night in Col-0 is caused by stomatal opening.

Diel patterns in stomatal opening in *Vitis vinifera*

The protocol was applied to detached grapevine leaves to investigate patterns in stomatal opening during the day and night.



Figure 8: Image of the experimental set up to analyse stomatal patterns in *Vitis vinifera* using PHENOPSIS. Detached leaves are emerged in artificial xylem sap and evaporation of the tube is avoided by putting adhesive paste around the petiole on the lid (coloured in red). Seventy leaves were widely spaced in the chamber to avoid shadow and contact during gravimetical measurement.

Long-term transpiration patterns of detached leaves

By using detached leaves, we were able to follow the transpiration dynamics on a high-throughput basis in controlled conditions using the PHENOPSIS automaton. A major advantage of using detached leaves is that changes in (specific) transpiration rate are expected to be the direct effect of stomatal behaviour as leaves were chosen as fully expanded (no growth anymore) and leaf movements were prevented by fixing the leaf on the tube. However, detaching leaves from the plant is expected to induce stress and senescence responses and therefore most detached leaf assays take no longer than 2-3 hours (Bazzaz et al., 1974; Green et al., 1998; Pantin et al., 2013). Although, excision and senescence responses may drastically influence the transpiration dynamic on the long term, it is not known to what extent and after how much time these processes appear on detached grapevine leaves. Therefore, an experiment was carried out on 70 detached leaves from 68 different genotypes derived from a biparental cross between Syrah and Grenache.

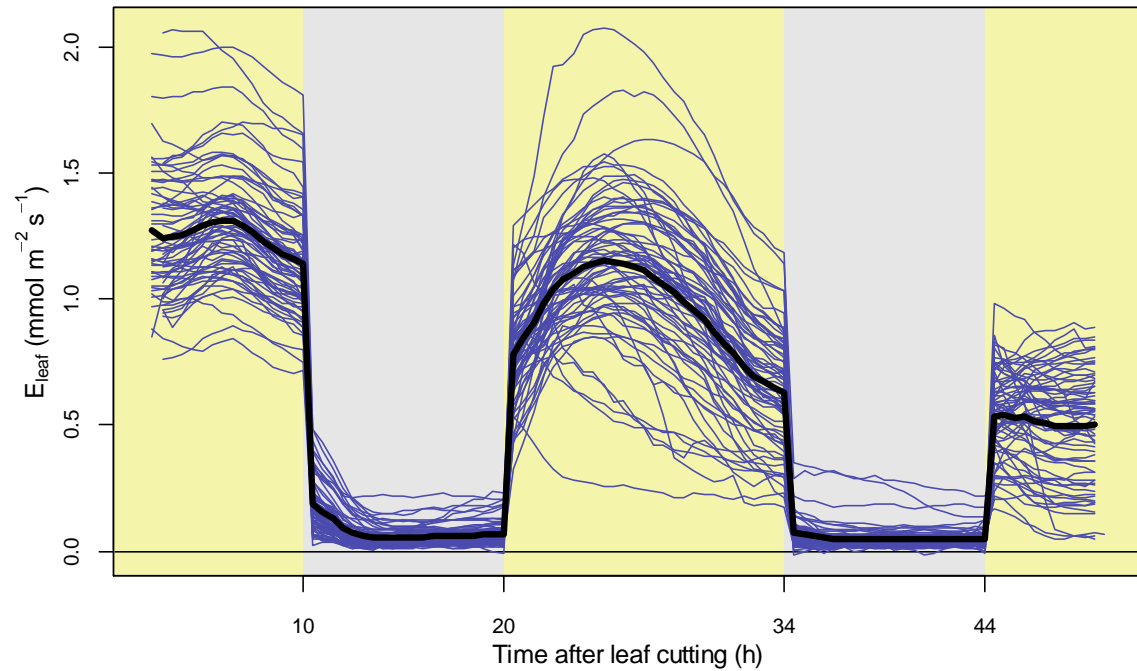


Figure 9: Transpiration patterns of detached grapevine leaves during 48-hours. Seventy leaves of 68 genotypes derived from a grapevine mapping population of Syrah and Grenache. The black line represents the average of all dynamics and grey period the night period.

The detached leaves weight losses were monitored during 48 hours to calculate the transpiration dynamic (Figure 9). After installation in the late morning (11 AM), transpiration increased till midday and decreased in the afternoon. Transpiration was drastically lowered at the onset of darkness, and remained almost stable during the rest of the night. After 18 hours in tubes, transpiration increased in response to light of the next day in all leaves and most leaves showed a typical diurnal transpiration pattern as in *Arabidopsis thaliana*: rapid increase (likely due to stomatal opening), steady increase until midday and decrease in the afternoon (Figures 7, 9). For a small number of leaves, transpiration rate increased normally but showed a steady decline afterwards suggesting that symptoms of stress appeared. These leaves exhibited mitigated responses of transpiration to darkness on the second night, suggesting a dysfunction of stomata. For most other leaves transpiration decreased as expected in responses to darkness on the second night, resulting in similar average nighttime transpiration levels as the preceding night. After 42 hours in tubes, the transpiration response to light was dampened in all leaves and did not show the similar dynamic as the day before suggesting the activation of stress and senescence processes influencing stomatal behaviour.

We conclude that detached leaves of grapevine could be used to monitor transpiration dynamics for at least 24 hours following excision before the arrival of severe stress symptoms. Using this method, the transpiration dynamic of a single leaf could be followed during day and night in controlled conditions at a high-throughput scale (70 leaves per PHENOPSIS chamber). As fully expanded grapevine leaves which had stopped growing were chosen for the analysis, differences in transpiration during the day and night corresponded almost exclusively to stomatal movements. Another advantage of the method is that other influences on transpiration, such as canopy structure or energy balance of the leaf were homogenised for all leaves by putting leaves in a similar orientation towards light. Our biological analysis could also focus on local signalling, disregarding possible influence of root signals. This allows us to analyse internal leaf processes that control stomatal behaviour.

Comparison of detached leaf and whole vine transpiration

We next examined to what extent isolation of the leaf from the rest of the plant did influence leaf patterns in transpiration compared to leaves still attached to the plant and whole-vine transpiration. We therefore compared the transpiration of detached leaves as described above with that of whole-plants and with gas-exchange measured on attached leaves using four genotypes. This experiment was repeated in two PHENOPSIS chambers: one with ambient CO_2 conditions (400 ppm) and a second with elevated CO_2 conditions (800 ppm). Elevated CO_2 was added to investigate how detached leaf transpiration responds to different environmental conditions. Five detached leaves, two whole-plants and one attached leaf per genotype were installed at the end of the afternoon in each of the chambers. First, the average transpiration at night per genotype was calculated for every method followed by correlation analysis for comparing the methods in both conditions (Figure 10A). Detached leaf transpiration significantly correlated with gas-exchange measurements and whole-plant transpiration. This suggests that detached leaves can be considered as representative of attached leaves on whole plants for comparing mean transpiration of various genotypes and environmental conditions.

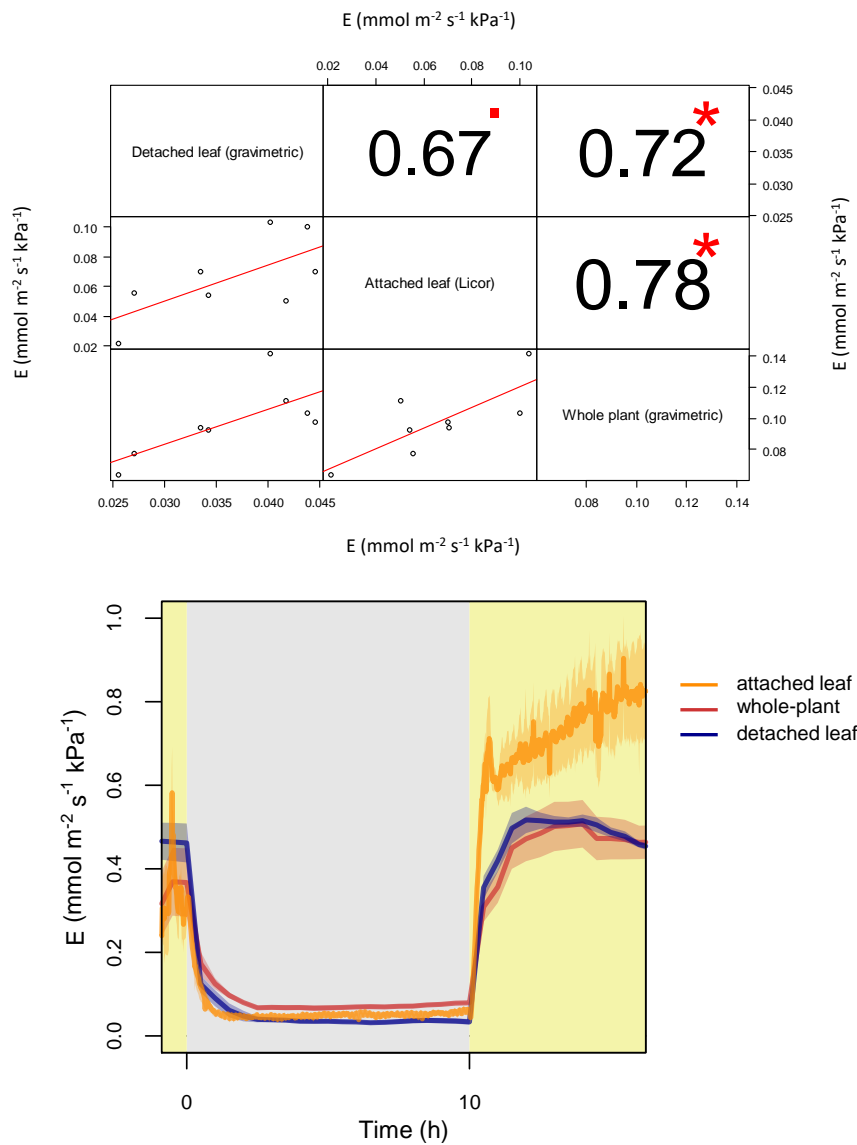


Figure 10: Comparison of detached leaf transpiration with whole-plant and attached leaf transpiration. A) Each point represents a single genotypic value for nighttime transpiration of either the

average of five detached leaves, a single leaf in a gas-exchange chamber or the average of two whole-plants for each of the two climate chambers, differing in the level of ambient CO₂. Whole-plants and detached leaves were installed at the end of the afternoon. Four genotypes were used for comparison analysis of transpiration. Correlations between average transpiration values at night with statistical tests using Pearson's correlation coefficients for both ambient and elevated CO₂ concentrations (symbols indicate $p < 0.05$ (asterisk) and $p < 0.1$ (square)). B) Average transpiration dynamic of four genotypes in ambient CO₂ conditions for detached leaves, whole-plants and attached leaves. Errors bars represent the standard error.

The average dynamic of transpiration rate for each method was calculated using the data of all genotypes under ambient CO₂ conditions (Figure 10B). Detached leaves had a slightly lower transpiration rate at night than whole-plants but during daytime no difference was observed between both methods. Several reasons may be responsible for this higher whole-plant transpiration at night, such as the contribution of younger and older leaves having generally a higher transpiration (Hopper et al., 2014) or spatial microclimate heterogeneity within whole plant canopy (Albasha et al., 2019) which depends on the plant position regarding ventilators and humidifiers. Moreover, nighttime transpiration rate of detached leaves was more stable than for whole plants where transpiration rate continuously increased. We cannot exclude an effect of leaf expansion for this increase in whole plants since we considered the surface area as fixed in our calculation. However, the fact that gas-exchange showed a similar increase in transpiration rate suggests that this increase could be due to stomatal reopening during the night. Stomatal reopening at night has been observed in field-grown grapevines (Rogiers et al., 2009) and in *Arabidopsis thaliana* (Figure 7). The apparent absence of stomatal reopening in detached leaves may be caused by the stress induced by excision or the AXS fed to leaves. However, stomatal reopening at night could also be observed in a test on detached leaves of three different genotypes suggesting that the absence of stomatal reopening is not necessarily due to our detached leaf protocol (Figure 7, Supplementary Figure 4). Stomatal reopening at night may also be influenced by photosynthesis on the day prior to assay (Easlon and Richards, 2009). We cannot guarantee that detached leaves obtained the same light treatment outside as whole-plants, possibly resulting variation of subsequent transpiration dynamics. Nevertheless, only a small reopening was observed at night in grapevine compared to *Arabidopsis thaliana* (Figure 7C). This suggests that variation in transpiration dynamics at night has a small influence on nighttime water loss in grapevine.

Genetic variation for diurnal stomatal patterns

Diurnal transpiration appeared as much more influenced by stomatal movements than nocturnal transpiration in grapevine (Figures 9). A progressive increase of transpiration rate until midday and gradual decrease in the afternoon has been observed in many plants in outdoors conditions closely following light intensity (Rogiers et al., 2009). This typical diurnal dynamic is also observed under steady light conditions (Lascève et al., 1997; van Wesemael et al., 2019) suggesting an internal signal that controls the dynamic of stomatal aperture. Indeed, we observed a similar diurnal dynamic of transpiration rate in whole plants of *Arabidopsis thaliana* (Figure 7), whole-plants of grapevine (Supplementary 5) and detached grapevine leaves (Figure 9). Several processes have been proposed for this 'dusk' anticipation, such as higher ABA sensitivity of the stomatal guard cells by circadian clock regulation (Pokhilko et al., 2013) or photosynthesis-derived signals from the mesophyll (Matthews et al., 2017), but the exact signal has not yet been identified. Therefore, a grapevine association panel was phenotyped for variation in diel transpiration dynamics on detached leaves to know if genetic variation existed for the level of decrease during the day.

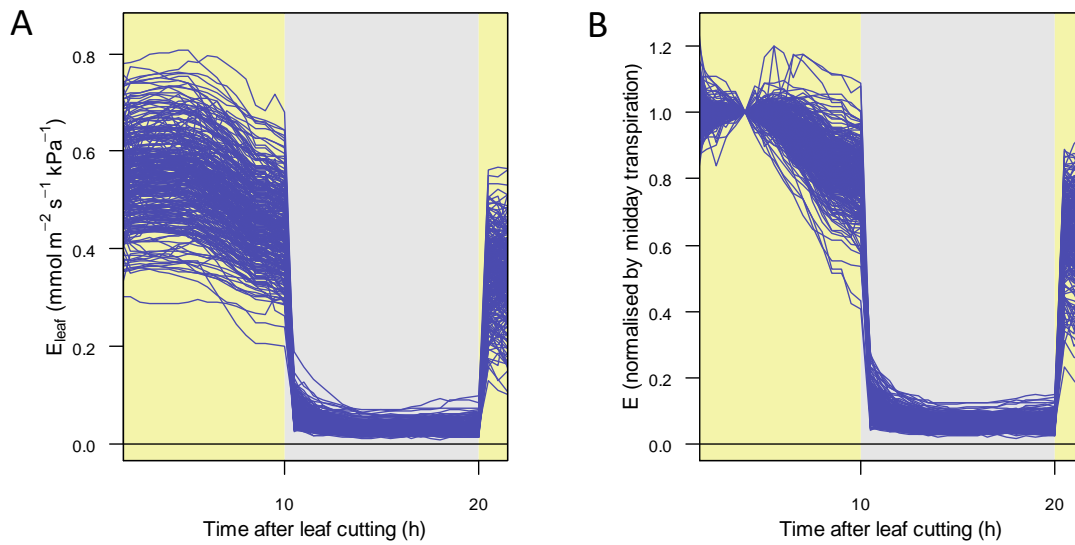


Figure 11: Genotypic diel transpiration dynamics on detached leaves for 279 genotypes of the grapevine association panel. A) Genotypic transpiration dynamics based on the average of five repetitions. B) Genotypic transpiration dynamics were normalised for their transpiration rate at midday to observe genotypic differences in stomatal closure in the afternoon.

Most genotypes increased transpiration rate until midday and decreased slightly in the afternoon (Figure 11A). Then, the diurnal transpiration pattern was normalised for its transpiration rate at midday (representing generally the peak of diurnal transpiration rate) to analyse the genetic differences in the relative decrease of transpiration during the ‘dusk’ anticipation (Figure 11B). We observed large variation among genotypes in the level of decrease in the afternoon (Figure 11B), possibly depending on the initial transpiration rate at midday.

Genotypes differed considerably in the level of stomatal closure in the afternoon from strong to almost no closure. The percentage of stomatal closure in the afternoon had a H^2 of 0.2, meaning that 20% of all phenotypic variation can be attributed to genetic variation. This confirms that our detached leaf assays can be used to detect genetic variation for dynamic transpiration traits in a grapevine association panel.

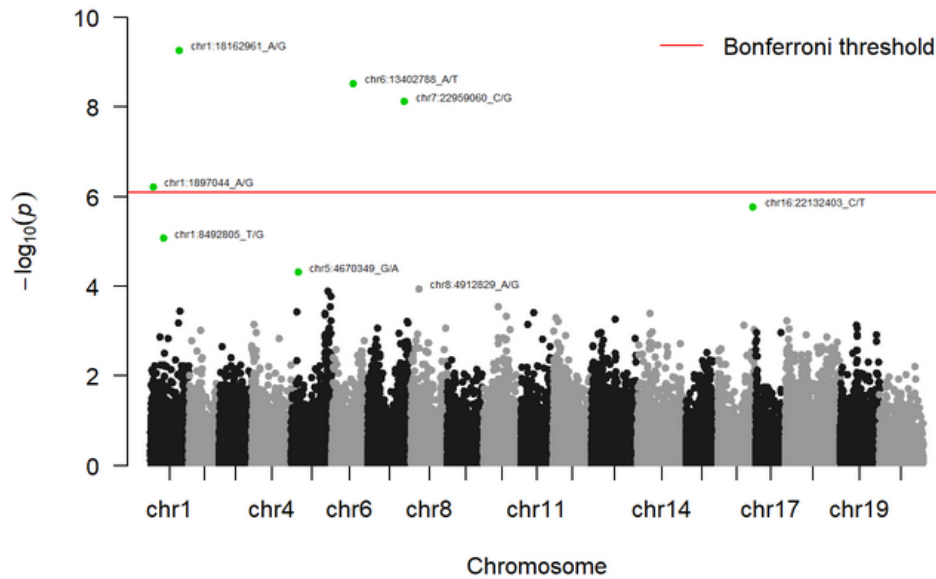


Figure 12: Manhattan plot of a GWAS by mlmm for the percentage of decrease in the afternoon.
The redline represent the Bonferroni threshold.

A GWAS was carried out by using mlmm and 90k SNPs on all genotypes of the association panel. After seven rounds of forward-backward marker selection we obtained at least four significant associations on chromosomes 1, 6 and 7 explaining the part of the variation in the decrease in the afternoon (Figure 12). Interestingly, the association on chromosome 7 was found for the average diurnal transpiration rate in detached leaves (Supplementary Figure 6). So by using a dynamic character derived from detached leaf transpiration we detected several new associations. Moreover, the colocalization of the association on chromosome 7 with average diurnal transpiration may suggest that the gene underlying this locus is controlling stomatal movements during the day.

Indoor and outdoor transpiration patterns

Transpiration dynamics in controlled conditions may show a similar pattern to outdoors. The level of outdoor transpiration is expected to be higher due to the influences of wind, higher VPD, higher light intensity and more extreme temperatures. Here, it was investigated to what extent transpiration dynamics in controlled conditions can be compared to outdoor plant transpiration dynamics. A comparison was made between the diel transpiration rate of six potted vines and of five detached leaves of cultivar Syrah in the climate chamber.



Figure 13: Comparison of detached leaf transpiration in PHENOPSIS with whole-plants outdoors. Diel transpiration pattern of detached grapevine leaves in controlled (blue) and whole-plant grapevine in outdoor (red) conditions. Five detached leaves of grapevine cultivar Syrah were cut in the morning in July and installed in controlled conditions (PHENOPSIS). In the same week the transpiration of six potted Syrah vines was measured gravimetrically in the vineyard. A) shows the course of photoactive radiation (PAR) in controlled and outdoor conditions, B) the VPD and C) the diel transpiration rate for both detached leaves in controlled conditions and whole-plant transpiration outdoors. The yellow and grey areas represent respectively the day and night in the climate chamber. Error bars represent standard error.

Light intensities were up to ten times higher outdoors than in the climate chamber (Figure 13A). The natural light regime outside increased from the onset of the day up to $2000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and

decreased till zero at the onset of the night. The climate chamber light regime was constant during the diurnal period and the start and end of the night matched the natural light conditions (Figure 13A). VPD was stable during the diel period in the climate chamber (1.5 kPa) whereas the outdoor VPD increased from the morning (1 kPa) until 12h (4 kPa) (Figure 13B). The VPD stayed constant during the afternoon and only decreased after complete sunset to 1.5 kPa. During the night, VPD decreased gradually from 1.5 kPa to 1 kPa (Figure 13B). As expected, outdoor transpiration rates were higher than detached leaves in the climate chamber during the diurnal period (Figure 13C). Transpiration on detached leaves increased during the day, reaching its maximum at midday (14h) and decreased in the afternoon. The outdoor transpiration rate increased strongly at the onset of the day, remained stable until 13h and decreased when light intensity decreases in the afternoon (Figure 13C). We have shown before that the decrease in transpiration rate in the afternoon is not completely dependent on the light intensity, but also partly by endogenous processes which are genotypic dependent (Figure 12). At the onset of the night, transpiration decreased strongly in both conditions and similar transpiration rate was observed between detached leaves in the climate chamber and whole-plants outdoor conditions. So, light intensity highly influenced the transpiration rate in outdoor conditions.

Discussion

Plant transpiration in controlled and uncontrolled conditions follows a dynamic behaviour during the 24-hour cycle of day and night. This behaviour does not strictly follow the changes in light conditions and variations in transpiration rates are still observed within the daytime and the nighttime while other climatic conditions are maintained as stable. This suggests that internal signals independent of environmental factors influence the level of plant transpiration. These signals may control transpiration via leaf movements and most importantly the opening of small pores called stomata. Stomatal opening exhibits a typical pattern during the day: they strongly open in response to light, gradually reopen till midday and decrease in the afternoon (Matthews et al., 2018). When the light is switched off in the climate chamber, stomata close abruptly, and can be followed by a gradual stomatal reopening till dawn. Although these stomatal dynamics highly influence diel water losses, the signals controlling them are not yet identified.

This knowledge gap is mainly due to the difficulty in measuring stomatal control of transpiration without confounding effects. Gas-exchange apparatus is probably the most precise way to measure the stomatal control on transpiration, but has the disadvantage to be a sensible apparatus which operates at a low throughput (one sample per machine with long equilibration time when changing the sample). Screening of genetic variation for transpiration dynamics may help to understand the internal signals controlling stomatal dynamics, but requires high-throughput methods to screen hundreds of genotypes. Transpiration dynamics can also be derived from gravimetric data (Cirelli et al., 2012). We developed a pipeline that is able i) to monitor weight losses in controlled conditions by using an automaton equipped with a mobile scale (Granier et al., 2006a) and ii) to automatically derive transpiration rate with a low time step resolution. The estimated transpiration dynamic may be used as a proxy for stomatal behaviour, although we show that leaf expansion in fast-growing plants as *Arabidopsis thaliana* in our conditions may influence considerably the dynamic of transpiration. For example, leaf expansion is higher at night than during the day (Pantin et al., 2011) and may account for part of the increases in nighttime transpiration rate during the night observed by whole-plant gas-exchange of *Arabidopsis thaliana* (Dodd et al., 2004, 2005; Klein et al., 2003, 2003; Lascève et al., 1997; Leymarie et al., 1998, 1999; Lü et al., 2012; Sierla et al., 2018) and *Helianthus annuus* (Auchincloss et al., 2014). Moreover, growth rate is genotype-dependent. For example, a starch synthesis mutant does

not have sugars at night to continue growing, leading to a lower nighttime growth compared to the day (Wiese et al., 2007).

Nevertheless, we show that leaf expansion during the night being approximately 8% variation in leaf area could not explain completely the gradual increases in nighttime transpiration (by almost 30%, Supplementary 1). Vertical leaf movements may increase transpiration by lowering the boundary layer conductance around leaves at the onset of the night. An effort should be done to better monitor plant growth and movements at a high frequency, for example by using laser scanning to produce a 3D plant (Dornbusch et al., 2012). However, the subsequent repositioning of the leaves during the remaining of the night is expected to induce a decrease in transpiration (Flood et al., 2016), contrary to our observations. Therefore, increase in transpiration rate during the night as observed in our experiments is most likely due to stomatal reopening. This stomatal reopening may be caused by a decreasing sensitivity to ABA (Pokhilko et al., 2013; Tallman, 2004), an increase of internal CO₂ produced by respiration (Fricke, 2019) or accumulation of sugars derived from the breakdown of starch. The increase in intracellular CO₂ concentrations by respiration is supported by the observation that *Eucalyptus camaldulensis* grown at high CO₂ concentrations increased stomatal reopening at night but did not increase starch levels at the end of the day (Resco de Dios et al., 2016). Higher ambient CO₂ concentration increases intracellular CO₂ concentration and stomata may open stomata to allow CO₂ diffusion into the atmosphere. Contrastingly, stomata are known to close during the day in response to high CO₂ concentration, and implies that the effect CO₂ on stomatal behaviour depends on the day or nighttime period.

Our developed protocol was applied to detached leaves of grapevine to investigate diel transpiration dynamics in this species. Although separated from the plant, detached leaves showed a remarkable responsiveness of stomata to light and darkness during 42-hours. Furthermore, averaged transpiration of detached leaves positively correlated with whole-plant and gas-exchange nighttime transpiration levels across genotypes and environments (ambient CO₂), allowing us to screen a grapevine association panel for genetic variation in transpiration dynamics. Only fully developed grapevine leaves were used, avoiding the effects of leaf expansion on transpiration dynamics as in whole plants of *Arabidopsis thaliana*. Detached leaves had a similar diurnal transpiration pattern to *Arabidopsis* and whole-grapevine plants in the climate chamber: transpiration increased from installation till midday and decreased in the afternoon. This suggests that these patterns are regulated at the leaf scale, independent of long-distance signals arising from the xylem such as sulphate (Malcheska et al., 2017; Tee, 2018), ABA (Dodd et al., 2008) or hydraulic signals (Jia and Zhang, 2008). At night, transpiration greatly decreased and remained almost stable, contrary to *Arabidopsis*. In the daytime, a decreasing dynamic of transpiration rate could be detected in the late afternoon and was selected for heritability analysis. We showed that our protocol could detect genetic variation for this trait and identified new genetic associations by GWAS. The level of decrease in the afternoon has been shown to be deregulated in a circadian clock mutant (Dodd et al., 2005) indicating the likely role of the circadian clock. The circadian clock is known to influence ABA signalling, which has been proposed to be involved in afternoon stomatal closure (Pokhilko et al., 2013; Tallman, 2004). The light regime during the day also strongly influence the daytime transpiration dynamic, with stronger decreases in the afternoon when exposed to the natural light regime (Matthews et al., 2018). Interestingly, genetic variation for photosynthetic activity exists during the diurnal cycle which may control stomatal behaviour in the afternoon (Flood et al., 2016, 2020). However, the signals linking photosynthesis and stomatal behaviour are not yet identified. Candidate gene analysis of the detected genetic associations will provide more insights in the genes controlling stomatal closing in the afternoon.

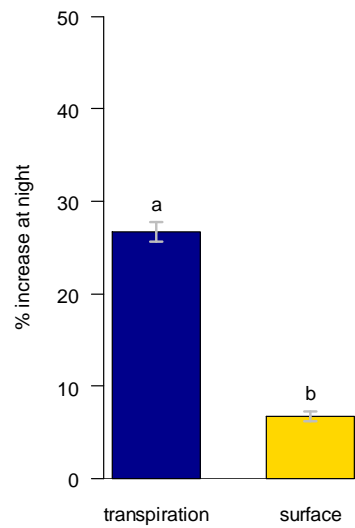
During the day, transpiration rate of whole-plant outdoors was higher than detached leaf transpiration rate in the climate chamber, probably due to stomatal opening in response to higher light intensity and regime. Stomata closely follow light intensity in the natural light regime and this behaviour is flattened in constant light conditions (Matthews et al., 2018). In our conditions, the transpiration rate outdoors was not always linked with light intensity. Especially in the early morning, transpiration rate increased strongly in whole-plants outdoors which may be a strategy of the plant to maximize photosynthesis in the cooler morning conditions, avoiding the more water-expensive photosynthesis in the afternoon when VPD was high. Stomata closed in both conditions completely in the absence of light, and showed a constant transpiration rate during the night. A similar transpiration rate pattern at night has been reported for potted vines in the field (Rogiers et al., 2009).

We showed that detached grapevine leaves can be used to detect genetic variation in diel transpiration dynamics. The next step will be to investigate the link between diurnal stomatal dynamics and its coordination with photosynthesis. Identification of underlying genes controlling the diurnal dynamics of transpiration rate may help to tune stomatal behaviour for an optimal use of water.

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Supplementary



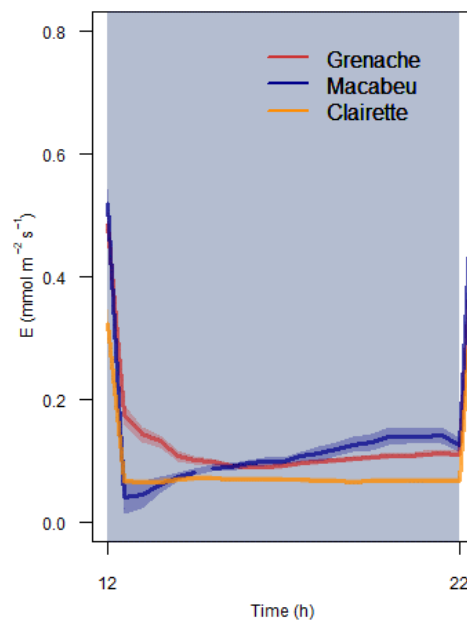
Supplementary figure 1: the percentage of increase in transpiration and surface between the start and end of the night in five plants of Col-0.



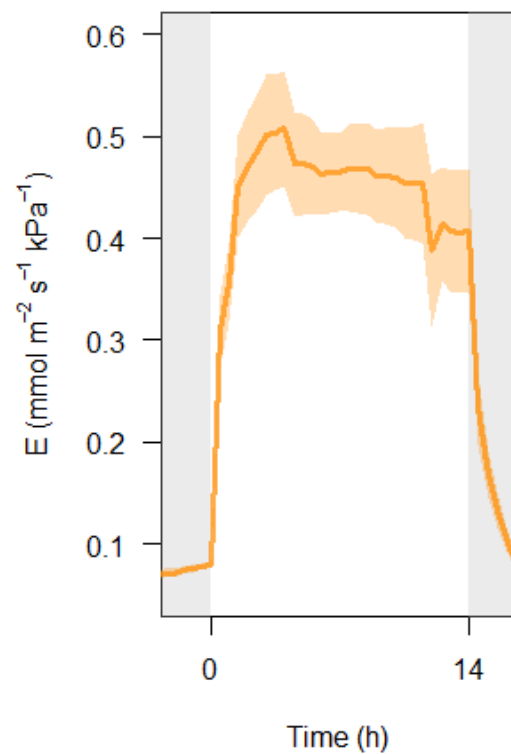
Supplementary figure 2: Heatmap showing the spatial variation of transpiration rates in the PHENOPSIS climate chamber on detached grapevine leaves during the day. A strong gradient can be observed from left to right.



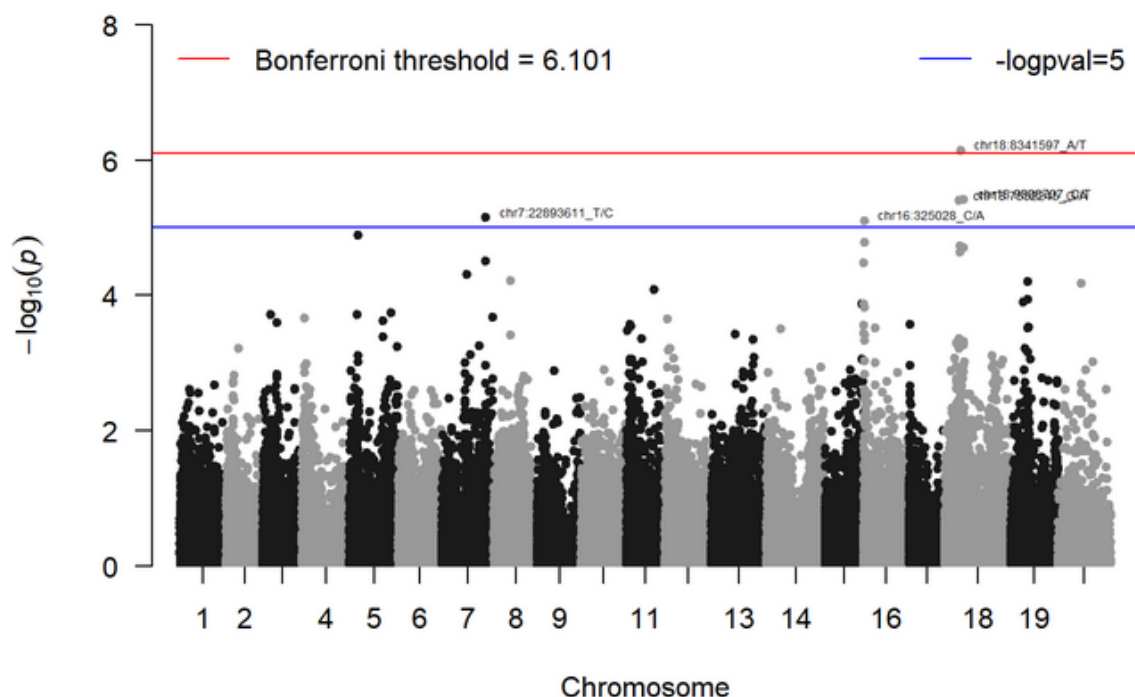
Supplementary figure 3: Heatmap showing the spatial variation of transpiration rates in the PHENOPSIS climate chamber on detached grapevine leaves during the night. A different gradient can be observed in comparison with transpiration rates during the day suggesting that daytime spatial variation is mainly influenced by the lights.



Supplementary figure 4: Nighttime transpiration patterns of detached leaves in the PHENOPSIS climate chamber for three different genotypes. Each treatment represents the average of five leaves.



Supplementary figure 5: Diurnal transpiration pattern of whole-plants in the PHENOPSIS climate chamber. The curve shows the average transpiration of four genotypes.



Supplementary figure 6: Manhattan of GWAS by GEMMA (procedure explained in chapter 3) on average daytime transpiration for all genotypes of the association panel.

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Chapter 3

A puzzling role for MALTOSE EXCESS 1 in stomatal dynamics

Outline

This chapter focuses on the GWAS result for average daytime transpiration on detached leaves in grapevine. A candidate gene was identified in the underlying region and the transpiration pattern of the *Arabidopsis* mutant was investigated in several environmental conditions to analyse the candidate gene action. Transpiration, biochemical and microscopic analysis of a collection of *Arabidopsis* mutants related to the candidate process was performed to dissect the signals between the mesophyll and guard cells modulating stomatal opening.

Contributors

This chapter was based on research I conceptualized with my supervisors Florent Pantin¹ and Thierry Simonneau¹. For data collection, I was helped at different stages by two interns I co-supervised, Jérémy Cortello (preliminary *Arabidopsis* experiment) and Lucille Roux¹ (grapevine GWAS experiment), the postdoctoral researcher Aude Coupel-Ledru¹ (grapevine GWAS experiment), as well as two research technicians, Gaëlle Rolland¹ (starch determination) and Myriam Dauzat¹ (Phenopsis monitoring). Florent Pantin¹ also collected data (gas exchange and luciferase experiment). I have performed all data analyses, after initial advices on grapevine quantitative genetics by Aude Coupel-Ledru¹, Timothée Flutre² and Agnès Doligez³. I contributed writing with Florent Pantin¹.

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Abstract

Stomata are minute pores at the leaf surface that open and close to poise gas exchange between the plants and the atmosphere. While CO₂ entry is required for photosynthesis, preventing excessive transpiration of water vapour is critical to avoid dehydration. Tailoring stomatal dynamics to better track the environmental fluctuations is a key strategy to improve water use efficiency in plants. Here we show that MEX1, a maltose transporter that is essential for nighttime starch metabolism, promotes stomatal opening according to an elusive mechanism. Quantitative genetics of transpiration dynamics in grapevine enabled us to identify *MEX1* as a candidate gene. Phenotyping the *Arabidopsis mex1* mutant revealed it is impaired in light-induced stomatal opening and endogenous nighttime reopening. Comparing *mex1* to other starch mutants indicated that the role of MEX1 in controlling stomatal movements extends beyond starch metabolism, but we could not conclude whether its action is cell-autonomous. Ultimately, MEX1 is likely to have a sharp but indirect effect on sugar and energy homeostasis in the guard cells, which regulates the solute transport processes that drive stomatal opening. Unlinking starch metabolism and stomatal movements offers a new target to breed crops with enhanced water use efficiency.

Introduction

Leaf stomata are tiny pores surrounded by pairs of guard cells that control carbon gain and water loss in plants (Hetherington and Woodward, 2003). Every day, stomata open to promote atmospheric CO₂ uptake when light is available to fuel photosynthesis, but this also generates transpiration of water vapour from the inner leaf tissues. When the light dims, stomata close preventing from inefficient use of water (Violet-Chabrand et al., 2017). At night, stomata show extensive closure, yet may reopen progressively in many species (Caird et al., 2007). Overall, the ability of stomata to track fast changes in irradiance or maintain low transpiration throughout the night enhances plant water use efficiency (Violet-Chabrand et al., 2017; Caird et al., 2007; Lawson and Blatt, 2014; Coupel-Ledru et al., 2016; Papanatsiou et al., 2019). As water becomes increasingly scarce with climate change, tailoring stomatal dynamics is a promising strategy to breed crops for drought tolerance.

Stomatal movements ultimately result from changes in osmotically-driven changes in guard cell turgor. Recent reports have revived the key role of starch and sugar metabolism and associated changes in osmolyte content in controlling light- and CO₂-induced stomatal dynamics (Horrer et al., 2016; Azoulay-Shemer et al., 2016; Azoulay-Shemer et al., 2018; Flütsch et al., 2020b; Flütsch et al., 2020a; Granot and Kelly, 2019). Here, following a genome-wide association study (GWAS) in grapevine, a candidate gene approach directed us to MEX1, a maltose transporter involved in nighttime starch breakdown, with previously unrecognized role on transpiration. Experiments on *Arabidopsis* revealed that MEX1 is involved in light-induced stomatal opening and endogenous reopening at night. Further analyses indicated that MEX1 uncouples starch metabolism from stomatal movements to a certain extent, yet the precise mechanism remains elusive. Our work sheds some light on the regulation of stomatal dynamics, providing a new avenue to breed crops for more efficient use of water.

Material and methods

Phenotyping and quantitative genetics in grapevine

Plant material and growing conditions

Plants for 279 cultivars of a grapevine diversity panel (Nicolas et al., 2016) were generated from cuttings obtained from the Vassal-Montpellier Grapevine Biological Resource Center (Marseillan-Plage, France). Non-grafted plants were grown outdoors in individual 3L pots with a mixture containing 30:70 (v/v) loamy soil and organic compost in the nursery at the Pierre Galet experimental vineyard of INRAE-SupAgro (Montpellier, France). Ten plants per cultivar were pruned to produce a single shoot, cut at a height of 3 meters and ferti-irrigated to a well-watered regime. Young flowers were systematically removed to avoid differences in vegetative and reproductive stages between genotypes.

Transpiration assay

The experiment was carried out on 2-years-old plants during summer 2019, from mid-June to mid-July. Fully developed leaves located at the 8th-12th phytomere were collected in the morning. Immediately after cutting, leaves were immersed in distilled water and their petioles were recut to avoid cavitation. Leaves were placed in tubes containing artificial xylem sap (AXS) containing 2 mol m⁻³ KH₂PO₄, 1 mol m⁻³ MES and 0.4 mol m⁻³ Ca(NO₃)₂ (Coupel-Ledru et al., 2016). Petioles were tightly sealed to the container cap to prevent direct evaporation of AXS. Every day, 70 leaves of random selected genotypes were collected in the midmorning and installed before midday in the PHENOPSIS automaton (Granier et al., 2006, https://www6.montpellier.inrae.fr/lepse_eng/M3P). This is a growth-chamber initially designed for the cultivation and monitoring of *Arabidopsis thaliana*. PHENOPSIS was used to expose grapevine leaves to stable daytime conditions for VPD (1.5 kPa), air temperature (25 °C) and light (180 μmol photons m⁻² s⁻¹) for nine hours. Doing that, we circumvented that daytime conditions influenced nighttime transpiration, as reported before (Easlon and Richards, 2009). Then, 10 night-hours were simulated by switching lights off, closely following the outdoor day/night cycle. Air temperature (25 °C) and VPD (1.5 kPa) stayed constant at night while leaves' weight losses were monitored every 30 minutes with a mobile Precisa XB 620M scale (Precisa Instruments AG, Dietikon, Switzerland). In the following morning, leaves were taken off the platform for epidermal structure and leaf surface analysis and replaced by a new batch of 70 leaves. This protocol was executed during one month in order to obtain six replicates from 5-6 plants per genotype.

GWAS

The genotypic values were calculated as in Chapter 3. Then, a genome-wide association study (GWAS) was conducted to identify associations between BLUPs of the association panel and 63,037 SNPs (with minor allele frequency, MAF < 0.05). SNP data were based on microarray and genotyping-by-sequencing (GBS) data followed by imputation (Flutre et al., 2020). The level of relatedness (kinship) between individuals was estimated based on SNPs with a linkage disequilibrium (LD) threshold of 0.5 using the kinship estimator of VanRaden (Flutre et al., 2020; VanRaden, 2008). The weak population structure as described in Nicolas *et al.* (Nicolas et al., 2016) was ignored. Quantitative trait loci (QTL) were detected using a SNP-by-SNP model implemented in GEMMA version 0.97 (Zhou and Stephens, 2012). A linear mixed model was used to test for every SNP: $BLUP(g) \sim 1\mu + M_{a,p}\beta_p + u + \epsilon$, where $M_{a,p}$ is a vector with genotypes at the p_{th} SNP and $\epsilon \sim N_N(0, \sigma_e Id)$ with N the normal distribution of dimension N , 0 a vector of zeros and Id the identity matrix of dimension $N \times N$. Relatedness was controlled by a random effect u , based on additive genetic relationships in a covariance matrix. The effect of SNPs was significant when the p value from the Wald test statistic was smaller than the Bonferroni threshold.

Physiological characterization in Arabidopsis

Plant material

Leaf starch metabolism in Arabidopsis is largely documented (Streb and Zeeman, 2012). All starch mutants used in this study were described previously and in the Col-0 background: *pgm-1* (AT5G51820; Caspar et al., 1985), *pgi1-1* (AT4G24620; Yu et al., 2000), *sex1-3* (AT1G10760; Caspar et al., 1991; Yu et al., 2001; Ritte et al., 2002), *amy3-2* (AT1G69830; Yu et al., 2005), *bam1* (AT3G23920; Fulton et al., 2008), *amy3-2 bam1* (AT1G69830 and AT3G23920; Horrer et al., 2016), *bam1 bam3* (AT3G23920 and AT4G17090; Horrer et al., 2016), *mex1-1* (AT5G17520; Niittylä et al., 2004), *dpe1-2* (AT5G64860; Critchley et al., 2001) and *dpe2-5* (AT2G40840; Chia et al., 2004). We also used starch mutants transformed with a starvation reporter construct consisting of the promoter of the starvation gene AT1G10070 fused to luciferase coding sequence (*ProStarv::LUC*; Graf et al., 2010) that was inserted in Col-0, *ss4-3* (AT4G18240; Crumpton-Taylor et al., 2013) and *isa1-1* (AT2G39930; Delatte et al., 2005) as described by Crumpton-Taylor (2010). We also used two mutants affected in a guard cell malate transporter, *abcb14-1* and *abcb14-2* (AT1G28010; Lee et al., 2008).

Growth conditions

The plants were grown in the Phenopsis phenotyping platform (Granier et al., 2006). Arabidopsis seeds were sown in 200-ml cylindrical plastic pots filled with a weighed amount of soil. To analyse all genotypes on the same day while reducing differences in plant architecture arising from contrasting growth rates among genotypes, the sowing date was adapted for each genotype based on a preliminary experiment: *pgm*, *sex1* and *mex1* were sown 42 days before the experiment; *bam3*, *bam3 bam3*, *pgi1*, *ss4-3 ProStarv::LUC*, *dpe1* and *dpe2* were sown 35 days before the experiment; and Col-0, Col-0 *ProStarv::LUC*, *bam1*, *amy3 bam1*, *amy3*, *isa1-1 ProStarv::LUC*, *abcb14-1* and *abcb14-2* were sown 28 days before the experiment. Plants were then grown under 12-h photoperiod at an irradiance of 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with constant air temperature (22 °C) and relative humidity (60%) during day and night. Each pot was weighed daily and its soil water content kept constant (1.4 g water g⁻¹ dry soil) by watering with a modified Hoagland solution.

Transpiration assay

Transpiration was measured by gravimetry. For the assay, the soil was covered with cellophane to prevent evaporation at the surface of the pot. 150 pots were weighed every 30 min during 48 h by a moving balance in the Phenopsis platform (Granier et al., 2006). Environmental conditions were held constant as during the growth period and were recorded in a database together with the weight data (Fabre et al., 2011).

The gravimetrical data were then processed using R scripts. Outliers were detected by analysing the studentized residuals of a local linear regression model of pot weight loss against time. Afterwards, the regression was run again and individual pot transpiration was calculated every 30 min as the fitted slope of the local linear relationship. The number of points taken into account at each time depended on the current position in the day/night cycle. Indeed, moving linear regression inherently buffers rapid changes such as occurring during the day/night transitions. Accordingly, weight values from the beginning of the night (resp. day) were never used to predict transpiration at the end of the previous day (resp. night). We therefore selected a varying time interval for the moving linear regression linear model: at the start of a day or night period, the interval was set to 90 min (3 points), gradually increased to 360 min (12 points) and gradually decreased back to 120 min (4 points) at the end of the same period. This varying interval allowed us to achieve a satisfactory trade-off between temporal resolution

at the day/night transitions and robustness elsewhere. The rate of water loss was corrected for plant growth to obtain transpiration per unit of projected leaf area. Leaf area was estimated by a linear or polynomial model (automatically selected for each plant depending on the fit quality) based on the daily projected surface measurements extracted by image analysis.

The final 24-h figures were obtained by averaging the transpiration values of the two consecutive days at each time point (technical replicates) for each plant (biological replicates, $6 \leq n \leq 8$). The 95% confidence intervals are presented. Nighttime stomatal reopening was quantified by subtracting the minimal value of nighttime transpiration from the transpiration value measured at the end of the night for every plant.

Starch

During the transpiration assay, leaf samples for starch analysis were obtained at the end of the day and night periods on distinct, adjacent plants. Four biological replicates were harvested per genotype at each time point. Approximately 40 mg of fresh leaf material per rosette was harvested, weighed and immediately frozen in liquid nitrogen. Starch content was analysed by enzymatic assay using spectrophotometric analysis of residual fraction of ethanol-water extracts (Hummel et al., 2010).

For iodine staining of starch, leaves were harvested and decolourised in 80% (v/v) ethanol. Excess of ethanol was removed by rinsing with water. Leaves were stained with a 30% (v/v) lugol solution for 30 min followed by a wash step of 30 min with water. The iodine staining was observed using an Olympus BX61 (60x, guard cells) and SZX16 (0.5x, whole leaves) microscope.

Gas exchange for maltose feeding assays

Gas exchange experiments were performed on xylem-fed detached leaves as described previously (Pantin et al., 2013b). Mature leaves of Col-0 plants were excised one hour before the end of the night period or during the daytime, and immediately recut under water. The petiole was then dipped in an Eppendorf containing water and the leaf was inserted into a 2x3 LED chamber of a Li6400-XT infrared gas analyser (Li-Cor Inc., Lincoln, NE, USA) connected to a Li610 dew-point generator (Li-Cor Inc.), and data were logged every 10 s. CO₂ was set at 400 ppm, block temperature at 22°C and reference dew-point temperature at 17°C. PAR was set at either 0, 50, 200 or 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ depending on the experiment. Once stomatal conductance had stabilized, water was changed against a 1 mM maltose solution. For the maltose effect at the dark/light transition stomatal opening, leaves were harvested at the end of the night, dipped into 1 mM maltose or water (control) for at least 2 h in darkness before proceeding to gas exchange analysis on a dark/light transition.

Luciferase assay

Plants were grown during two weeks in soil at 20 °C with 12-h photoperiod. One day before bioluminescence imaging, 0.5 ml of a luciferin solution (0.8 mM D-Luciferin Firefly, 0.02% 827 Triton X-100) was sprayed on each plant. Plants were assayed for bioluminescence every hour from the middle of the night to an eight-hour prolongation of the night. Luciferase signal was measured and quantified using the NightOwl imaging system with WinLight software (PerkinElmer, Waltham, MA, USA).

Statistics

To test for significant differences between genotypes, we used the Kruskal-Wallis non-parametric test at the 5% level followed by multiple comparison of means through a t-student test on the ranks using R 3.4.1 (R Development Core Team, 2017).

Results

Performing a GWAS on transpiration dynamics in grapevine unveils a candidate gene involved in nighttime starch metabolism

The march of stomatal movements occurs every day and night. To gain insight in the temporality of the underlying processes, we scrutinized transpiration dynamics in grapevine, a perennial crop with proven genetic variability in day- and nighttime transpiration (Coupel-Ledru et al., 2016). For this purpose, we adapted a high-throughput phenotyping platform devoted to Arabidopsis (Granier et al., 2006) so as to monitor the diel (24-h) kinetics of transpiration on 70 grapevine detached leaves with a 30-min resolution in controlled environmental conditions (Figure 1A). The devised protocol enabled us to phenotype a grapevine association panel with 279 genotypes (Nicolas et al., 2016; Flutre et al., 2020) for diel transpiration. Collecting leaves shortly after dawn allowed stomatal opening to be already stimulated before leaf excision, and limited the risk of cavitation that augments as the day progresses. The mean genotypic pattern revealed that leaves had already achieved substantial transpiration rate upon transfer to the phenotyping chamber, before showing a gentle increase to reach a plateau of about $0.55 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ kPa}^{-1}$ in the middle of the daytime, and then a gradual drop to about $0.45 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ kPa}^{-1}$ at the end of the daytime (Figure 1B). This pattern was due to endogenous stomatal movements because the environmental conditions were held constant. When the light switched off, stomata closed and transpiration rapidly decreased to $0.04 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ kPa}^{-1}$ on average and remained essentially stable throughout the night. When the light was switched on again, stomata reopened and transpiration increased rapidly, yet to a lower extent than in the previous day (Figure 1B). Overall, monitoring diel transpiration on detached leaves produced reliable stomatal dynamics.

We then performed a GWAS using the transpiration cumulated during the first day and night, which revealed several SNPs associated with diel transpiration (Figure 1C). One of them on chromosome 16 held our attention since it was located at 10 kb of *MALTOSE EXCESS 1* (*MEX1*), a gene involved in another diel process, the metabolism of transitory starch in leaves (Figure 1D). Transitory starch accumulates in the leaf chloroplasts during the day and is broken down at night, which buffers the diel carbon status of the leaves despite the fluctuations in light availability (Stitt and Zeeman, 2012; Streb and Zeeman, 2012). The major product of chloroplastic starch breakdown at night is maltose, which is generated by the action of β -amylases and then exported to the cytosol by the *MEX1* chloroplastic transporter (Niittylä et al., 2004; Weise et al., 2004; Weise et al., 2005). For convenience, the pathway described in Stitt and Zeeman (2012) is shown on Figure 2A. Interestingly, inspection of the mean kinetics for the different allele combinations at the SNP revealed that the homozygous genotypes for the allele chr16:325028_C/A conferred a global increase in daytime transpiration, while no significant effect was observed at night (Figure 1D). Moreover, period-specific GWAS allowed us to retrieve the same SNP in the daytime (Fig 1E), while it could not be detected at night (Westgeest et al., in prep., see chapter 3).

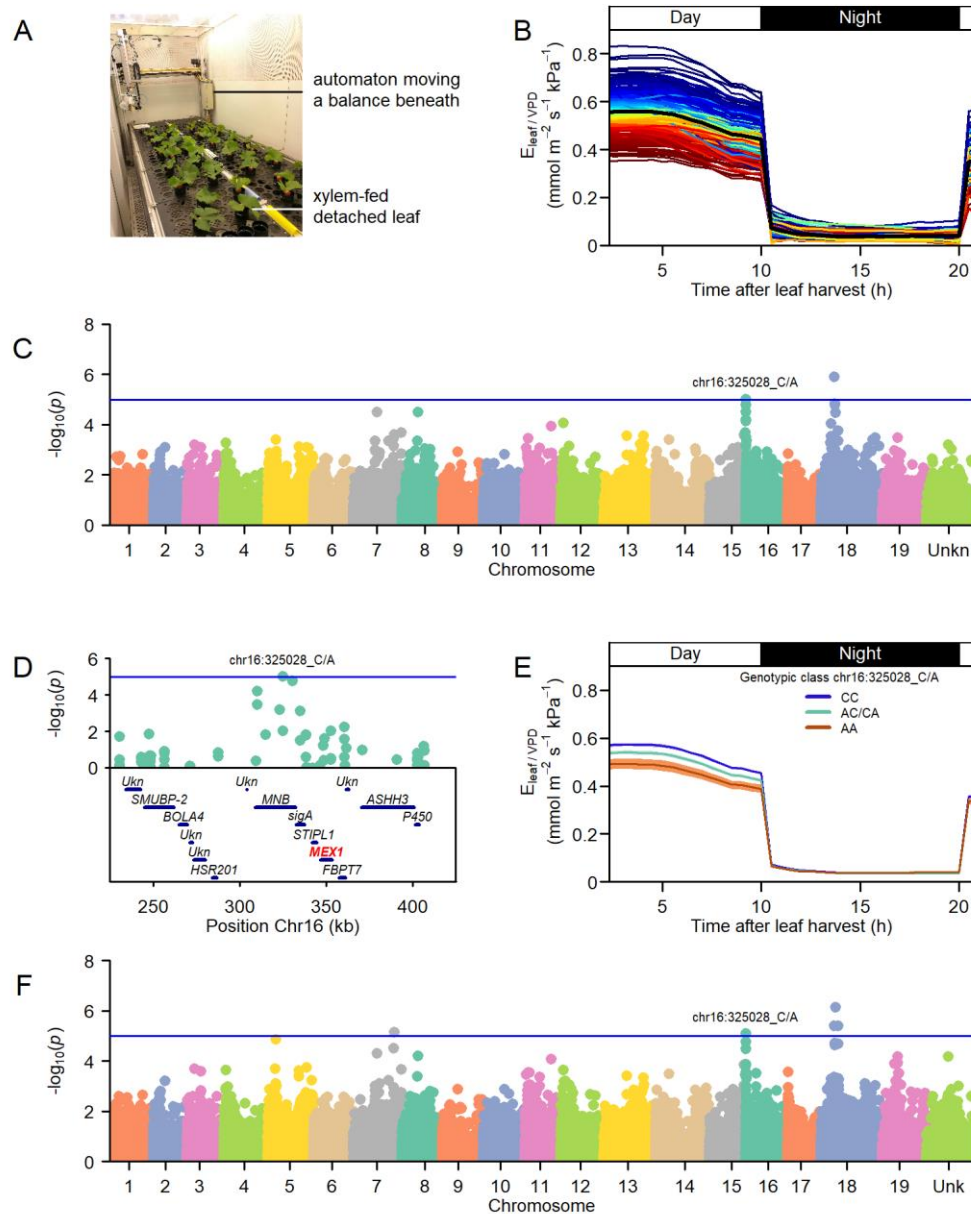


Figure 1. GWAS-driven identification of a candidate gene for diel transpiration in grapevine. A. Photograph of the phenotyping setup developed to monitor the diel transpiration in grapevine detached leaves at a high throughput. B. Average diel dynamics across all genotypes. C. GWAS for the first day and night (20 h) of the transpiration dynamics. The significant SNP highlighted on chromosome 16 (325028_C/A) was 10 kb away from MEX1. D. Candidate genes in the 200 kb region underlying the SNP chr16: 325028_C/A. MEX1 is coloured in red. E. Average diel dynamics for the different alleles at the SNP on chromosome 16. F. GWAS for the first day only identifies the same SNP.

A role for MEX1 and starch metabolism in stomatal opening

To assess whether MEX1 affects diel transpiration, we used our phenotyping pipeline on Arabidopsis whole-plants (Figure 3A) and compared the wild-type Col-0 with the *mex1* mutant, which over-accumulates maltose in the chloroplast and cannot fulfil starch breakdown at night (Niittylä et al., 2004; Lu et al., 2006; Figure 2A-C). Again, we observed transpiration patterns that could be ascribed to rapid stomatal movements at the day/night transitions and to slower, endogenous stomatal

rhythms otherwise (Figure 3B). In Col-0, stomata opened rapidly when the light switched on, continued to open until the middle of the day and then partly closed until the end of the day. When the light switched off, stomata closed rapidly but incompletely, and then reopened gradually throughout the night (Figure 3B). In *mex1*, stomatal opening at dawn was largely slowed down, with the time required to reach half of the amplitude being delayed by about 2 h, while the maximal rate of daytime transpiration was not affected (Figure 3B). Upon darkness, the rate of stomatal closure was only marginally impaired, while the gradual nighttime reopening was abolished (Figure 3B). Thus, MEX1 appears to mainly accelerate stomatal opening, both in the daytime and in the nighttime. Similar phenotypes had already been reported for *pgm* (Lascève et al., 1997), a mutant that cannot synthesise starch due to deficiency for chloroplastic phosphoglucomutase (Caspar et al., 1985; Figure 2A-C). PGM converts glucose-6-phosphate (Glc6P) into glucose-1-phosphate (Glc1P), which is the substrate for ADP-glucose pyrophosphorylase (AGPase). The latter produces ADP-Glucose (ADPGlc), the precursor for starch synthesis (Streb and Zeeman, 2012). We confirmed that *pgm* stomata open more slowly at dawn and do not reopen at night in our conditions (Figure 3C). Moreover, both dawn opening and nighttime reopening were affected in *sex1* (Figure 3C), a mutant defective in the plastidial α -glucan water, dikinase (GWD1) that is responsible for the first step of glucan phosphorylation necessary for starch degradation (Caspar et al., 1991; Yu et al., 2001; Ritte et al., 2002). In the *sex1* mutants, daytime starch synthesis proceeds without nighttime degradation, resulting in a severe starch-excess phenotype (Streb and Zeeman, 2012; Figure 2A-C). Thus, the capacity for starch mobilization rather than starch content *per se* seems to promote stomatal opening.

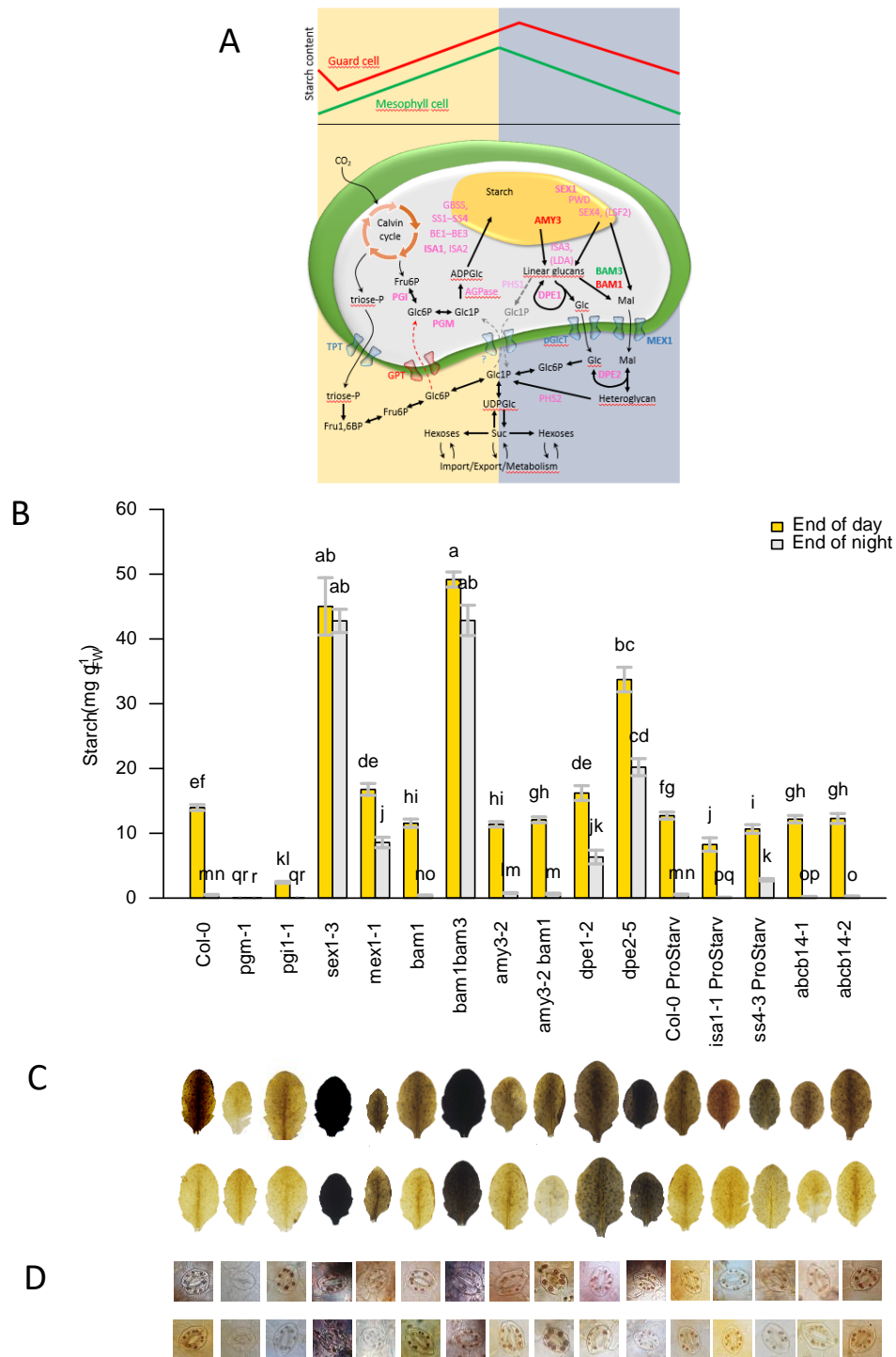


Figure 2. The starch pathway in Arabidopsis and characterization of corresponding mutants. A. The synthesis and degradation pathway of transitory starch in mesophyll cells of Arabidopsis leaves

(adapted from Stitt and Zeeman, 2012). B. Leaf starch content determined at the end of the day and night periods. Error bars are means \pm SE ($n = 4$). Letters denote significant differences after a Kruskal-Wallis test ($\alpha = 5\%$) followed by multiple comparison of mean ranks. C,D. Iodine staining of starch on entire leaves (C) or individual guard cells (D) at the end of the day (top) or night (bottom). Note that the *abcb14* mutants are not part of the starch pathway (see text).

Dial stomatal dynamics are not sustained by cell-autonomous starch degradation

Starch and sugar metabolism have long been postulated as important processes in stomatal movements (Granot and Kelly, 2019), yet the spatiotemporal hierarchy of events that connect carbon metabolism and guard cell dynamics remains a matter of debate. Starch breakdown, like photosynthesis, produces sugars that may trigger stomatal opening by providing the signal, energy or osmotica required for turgor build-up of the guard cells (Santelia and Lawson, 2016; Daloso et al., 2017; Lawson and Matthews, 2020). Interestingly, the turnover of transitory starch in the guard cells is slightly out of phase compared to that in the mesophyll cells (Santelia and Lunn, 2017). Notably, guard-cell starch degradation persists during the first hour of the day to support sugar-induced stomatal opening at dawn, through the guard-cell specific and synergistic action of the isoforms α -amylase AMY3 and β -amylase BAM1 (Horrer et al., 2016; Flütsch et al., 2020b). However, while sugars in the guard cells may come from cell-autonomous starch metabolism and photosynthesis, they may also be imported from the mesophyll cells. Most recent research supports the idea that guard-cell starch degradation sustains blue-light induced stomatal opening, whereas mesophyll-derived sugars supports red-light induced stomatal opening (Flütsch et al., 2020b; Flütsch et al., 2020a). However, it remains elusive whether cell-autonomous starch metabolism mediates nighttime stomatal reopening.

To ascertain the current framework for stomatal opening at dawn and tackle the question of nighttime reopening, it was necessary to differentiate between starch from the guard cells and the mesophyll cells. In our study, the starch phenotype observed in the guard cells was consistent with that in the mesophyll cells for the starch-free *pgm* and the starch-excess *sex1* (Figure 2B-D), as previously reported (Lascève et al., 1997; Horrer et al., 2016; Azoulay-Shemer et al., 2018). These mutants are therefore not suitable to distinguish between both cell types. However, intriguingly, the guard cells of *mex1* appeared essentially starch-free, in contrast with its starch-excess mesophyll cells (Figure 2B-D). Thus, it was not possible to speculate whether stomata of *mex1* do not reopen at night because sugars borne from starch degradation remain trapped as maltose in the chloroplasts of mesophyll cells, or because there is no starch to be broken down in the guard cells. The opposite starch pattern is known from the *pgi1* mutant, which is defective in the plastidial phosphoglucose isomerase that produces Glc6P from Fru6P upstream of PGM (Yu et al., 2000; Figure 2A): while starch synthesis is largely impaired in *pgi1*, some cell types having an active glucose 6-phosphate/phosphate translocator (GPT) bypass the PGI step by importing Glc6P from the cytosol (Niewiadomski et al., 2005). Accordingly, *pgi1* is essentially starch-deficient in the mesophyll cells, but retains starch synthesis e.g. in the guard cells, the bundle sheath cells and some adjacent cells (Tsai et al., 2009; Kunz et al., 2010; Azoulay-Shemer et al., 2016), and we confirmed this pattern (Figure 2B-D). While the transpiration baseline was higher in *pgi1* than in Col-0, similar dynamics were observed in the daytime, and nighttime reopening was reduced but not abolished (Figure 3D). Again, we were unable to assign a cellular origin for this intermediate nighttime phenotype, because several cell types were able to accumulate starch, resulting in a significant yet reduced daytime accumulation when measured at the bulk-leaf level (Figure 2B-D). We then examined the double mutant *amy3 bam1* that is severely impaired in starch degradation in the guard cells but has intact starch turnover in the bulk leaf (Figure 2B-D). The diel dynamics of transpiration of *amy3 bam1* were indistinguishable from that of Col-0 (Figure 3D), just like

the single mutants (Figure S1). This result confirmed that the contribution of guard-cell starch degradation to stomatal opening at dawn is specific to blue light and overridden by photosynthetic red light (Lascève et al., 1997; Flütsch et al., 2020b; Flütsch et al., 2020a), and also showed that cell-autonomous starch breakdown is not required for nighttime stomatal reopening. Instead a fraction of sugars produced by nighttime starch degradation in the mesophyll could progressively diffuse into the apoplast, reach the guard cells and sustain stomatal reopening as the night progresses. Similarly, sugars remaining in the apoplast at dawn could promote light-induced stomatal opening.

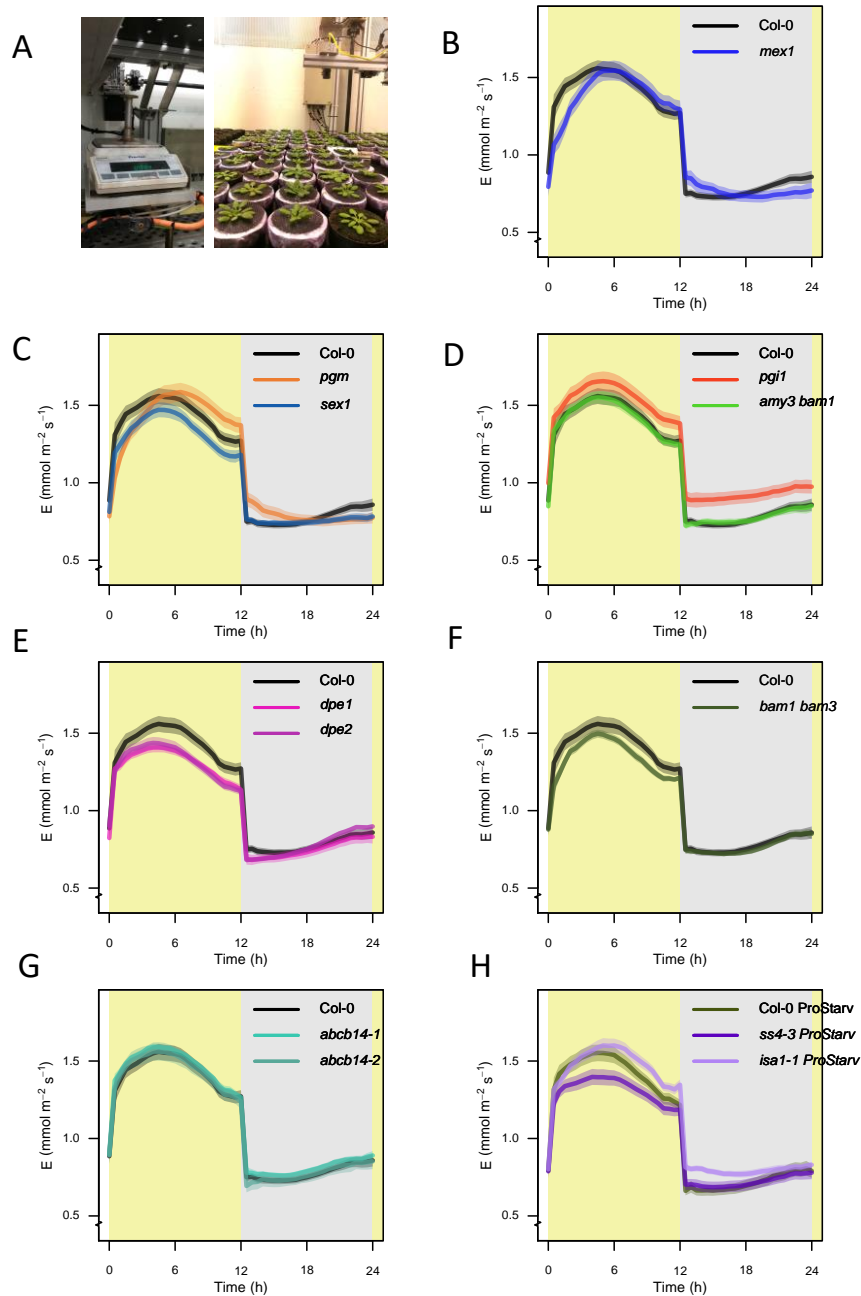


Figure 3. Diel transpiration dynamics in the starch and malate mutants. A. Overview of the phenotyping system including the mobile balance (left) and the plants with soil covered with cellophane to prevent evaporation and a black grid to facilitate plant detection through image analysis (right). B–H. Diel transpiration dynamics of the wild-type Col-0 (B–G) or Col-0 ProStarv::LUC (H) compared to *mex1* (B), *pgm* and *sex1* (C), *pgi1* and *amy3 bam1* (D), *dpe1* and *dpe2* (E), *bam1 bam3* (F), *abcb14-1* and

abcb14-2 (G), and *ss4 ProStarv::LUC* and *isa1 ProStarv::LUC* (H). The 95% confidence interval is shown ($6 \leq n \leq 8$). These data correspond to the average of the first two control days presented on Figure 4 and S5.

Neither sugars, maltose or malate from the mesophyll can account for the diel dynamics of transpiration

While the latter scenario fits well with the current model whereby stomatal response to light requires mesophyll-derived sugars (Flütsch et al., 2020a; Flütsch et al., 2020b), it is not consistent with the fact that shortly after the onset of illumination, massive amounts of soluble sugars are found in the leaves of starch mutants such as *mex1* (Niittylä et al., 2004), *pgm* (Caspar et al., 1985; Gibon et al., 2004) or *sex1* (Caspar et al., 1991), making it unlikely that the lack of exported sugar impedes stomatal opening during several hours in these genotypes. Alternatively, one could argue that the excess of sugars may prevent stomatal opening in those genotypes, as guard-cell sucrose, for instance, could open or close stomata (Kelly et al., 2013; Daloso et al., 2015; Lugassi et al., 2015; Daloso et al., 2016b; Daloso et al., 2016a). Nevertheless, the *pgi1* mutant also accumulates high levels of sugars shortly after dawn (Kunz et al., 2010) and yet shows similar stomatal dynamics as in the wild-type (Figure 3D).

Instead of total sugars, a specific molecule that is normally transported from the mesophyll to the guard cells, where it promotes stomatal opening, may be lacking in the starch mutants. Maltose, which is trapped in the chloroplasts of *mex1* (Niittylä et al., 2004), could be such molecule. Indeed, maltose transport is detected in phloem exudates of Arabidopsis leaves (Lu et al., 2006), and exogenous maltose added on epidermal peels gently enhances stomatal opening (Dittrich and Mayer, 1978). To test this hypothesis, we first manipulated starch turnover independently of maltose by using *dpe1*, a mutant affected in the chloroplastic disproportionating enzyme 1 that uses short malto-oligosaccharides during starch degradation to release glucose (Critchley et al., 2001), which is further exported to the cytosol by the glucose transporter pGlcT (Cho et al., 2011; Figure 2A). The glucose route is parallel to the maltose route and is thought to be minor (Streb and Zeeman, 2012). Here, blocking the glucose pathway in *dpe1* affected starch turnover similarly as in *mex1* (Figure 2B-C), but resulted in contrasting stomatal dynamics: the rate of dawn opening and nighttime reopening were similar as in the wild-type, while the magnitude of daytime transpiration was reduced (Figure 3E). Thus, different blockages in the starch pathway may yield the same starch turnover but different effects on maltose and transpiration. We then phenotyped *dpe2*, a mutant affected in the cytosolic disproportionating enzyme 2 that uses the maltose exported by MEX1 to release glucose (Streb and Zeeman, 2012; Figure 2A). The *dpe2* mutant has a starch-excess phenotype (Figure 2B-C), and accumulates extremely high levels of maltose in the cytosol (Chia et al., 2004; Lu and Sharkey, 2004; Weise et al., 2005; Lu et al., 2006). If maltose were to diffuse out the cytosol of mesophyll cells to enforce stomatal opening, we would expect that the dramatic increase in cytosolic maltose of *dpe2* would strongly enhance its transpiration dynamics. However, we observed a moderate enhancement of nighttime reopening, while the amplitude of daytime transpiration was reduced (Figure 3E). We further analysed the *bam1 bam3* mutant, where the two main β -amylases of the leaves are knocked down, resulting in a severe starch-excess phenotype in the mesophyll and in the guard cells (Figure 2B-D), and a lack of maltose production (Fulton et al., 2008; Horrer et al., 2016). The diel transpiration of *bam1 bam3* was similar to that of Col-0, except a modest reduction in the maximal daytime transpiration (Figure 3F). Finally, monitoring gas exchange in detached leaves fed with 1 mM exogenous maltose revealed no effect on stomatal dynamics, either in the dark, in the light, or during

dark/light transition (Figure S2) – yet we could not determine whether maltose that was fed through the transpiration stream had actually reached the guard cells. We conclude that mobile maltose is not responsible for enhancing stomatal opening. An alternative candidate could be malate, which is thought to coordinate mesophyll photosynthesis and stomatal behaviour (Araújo et al., 2011; Daloso et al., 2017). However, two independent mutants impaired in ABCB14, a guard cell malate importer (Lee et al., 2008) with unaltered starch turnover (Figure 2B-D), showed a diel transpiration indistinguishable from the wild-type (Figure 3G). Overall, while impairments in mesophyll starch metabolism had various effects on transpiration dynamics, none of total sugars, maltose or malate could provide a clear-cut explanation for all the phenotypes observed.

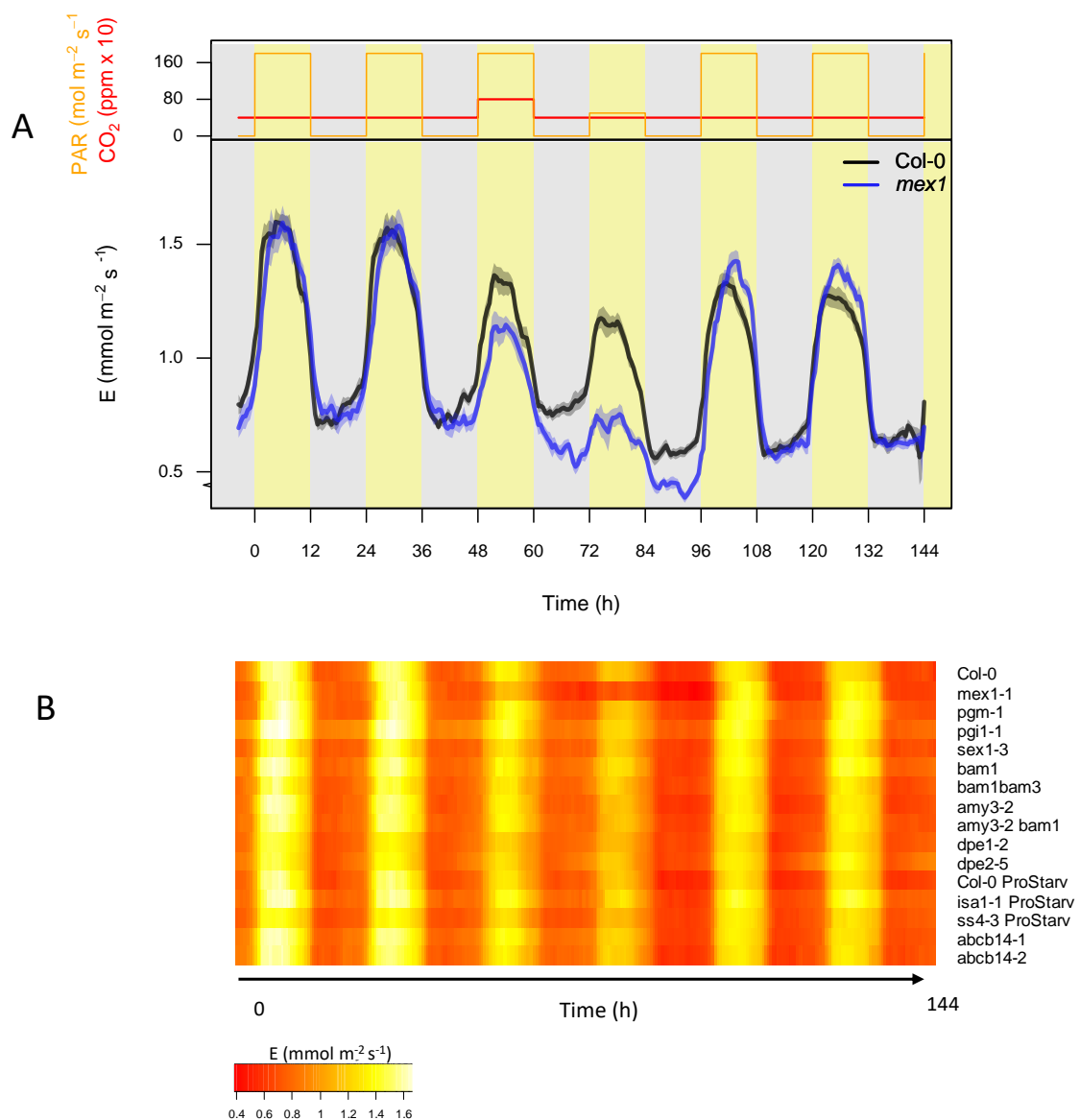


Figure 4. Effects of elevated CO₂ and low light on the diel transpiration dynamics. After two control days at 400 ppm and 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$, atmospheric CO₂ was enhanced to 800 ppm in the daytime and then returned to 400 ppm at the beginning of the following night. On the day after, irradiance was reduced to 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were then given two days of recovery. A. Kinetics of Col-0 compared to mex1. The 95% confidence interval is shown ($6 \leq n \leq 8$). B. Heatmap depicting the mean kinetics of

all genotypes. The individual kinetics of each genotype compared to its wild-type can be found on Figure S5.

Leaf carbon status does not correlate with stomatal movements

Among the diversity in transpiration dynamics, the most severe phenotypes we observed in *mex1*, *pgm* and *sex1* seem to be associated with the most acute metabolic statuses, corresponding to carbon starvation (Gibon et al., 2004; Bläsing et al., 2005; Pantin et al., 2011; Stitt and Zeeman, 2012; Pantin et al., 2013a). To test the idea that carbon starvation may somehow prevent stomatal opening, we used a starvation reporter construct (Graf et al., 2010) that was inserted into two independent starch mutants (Crumpton-Taylor, 2010). First, the *ss4* mutant, which is affected in starch synthase 4 that is required for starch granule initiation, displays reduced starch synthesis and incomplete degradation (Roldán et al., 2007; Crumpton-Taylor et al., 2013; Lu et al., 2018). Second, the *isa1* mutant, which is impaired in the debranching enzyme isoamylase 1 that contributes to the structure of starch, accumulates phytoglycogen rather than only starch and shows premature exhaustion of its reserves during the night (Delatte et al., 2005; Wattedled et al., 2005). Accordingly, the reporter indicates a constitutive yet low starvation at night in *ss4*, a rapid increase in starvation 2 h before the end of the night in *isa1*, while no starvation is detected in the wild-type as long as the night is not extended (Crumpton-Taylor, 2010; Figure S3). Contrary to the hypothesis, nighttime stomatal reopening was detected in *ss4* but was abolished in *isa1* (Figure 3H). Moreover, other conditions inducing carbon starvation, like low CO₂ or autophagy, are associated with stomatal opening, not closure (Yamauchi et al., 2019). Thus, carbon starvation *per se* is unlikely to cause impeded stomatal movements.

To further evaluate the effect of varying carbon status on diel transpiration, we then subjected all the mutants to elevated CO₂ and then low light. After two control days at 400 ppm and 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$, atmospheric CO₂ was doubled to 800 ppm in the daytime (Figure 4A). In the wild-type, this treatment resulted in a 25% enhancement in daytime starch accumulation (Figure S4) and a 15% decrease in daytime transpiration (Figure 4A), consistent with the closing effect of elevated CO₂ on stomata (Zhang et al., 2018). At night, CO₂ was returned to 400 ppm (Figure 4A). The remaining starch at the end of the night almost tripled (Figure S4), indicating carbon satiation, while stomata closed to a lesser extent resulting in an enhancement of nighttime transpiration and a reduction in stomatal reopening (Figure 4A). This treatment was followed by a daytime at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which dramatically reduced daytime starch synthesis and tended to exhaust the remaining starch at the end of the night (Figure S4), suggesting carbon starvation. Daytime transpiration decreased compared to the control day, consistent with stomatal response to irradiance (Shimazaki et al., 2007), to a more severe extent than for elevated CO₂, while nighttime transpiration was reduced and stomatal reopening eliminated (Figure 4A). Finally, two days of recovery showed that diel transpiration retrieved its original dynamics, with a global trend of transpiration attenuation as the rosette developed (Figure 4A). Across all the mutants we phenotyped, *mex1* was the most dramatically affected (Figure 4A-B). At elevated CO₂, daytime transpiration was reduced by 25% while stomata were continuously closing during the following night at 400 ppm (Figure 4A). Under low light, stomata of *mex1* barely opened, and continued to close in the following night (Figure 4A). Diel transpiration immediately recovered upon return to the original growth conditions (Figure 4A). The other mutants, including *pgm* and *sex1*, essentially responded as the wild-type (Figure 4B, Figure S5). While we could not interpret the whole kinetics, these results clearly demonstrated that the role of MEX1 in stomatal opening is not directly linked to starch synthesis or degradation.

Discussion

***MEX1* as a strong candidate for an SNP of diel transpiration in grapevine**

Understanding the determinants of day and night transpiration, and associated stomatal behaviour, is crucial to design strategies for breeding plants with enhanced water use efficiency (Lawson and Blatt, 2014; Coupel-Ledru et al., 2016). Using GWAS in grapevine, we uncovered SNPs involved in the control of diel transpiration. One of them on chromosome 16 was very close to the gene coding for *MEX1*, the chloroplastic transporter through which maltose, the major product of transitory starch breakdown, is exported at night to sustain plant's metabolic needs in the absence of photosynthesis. Unexpectedly, the SNP was specifically associated with daytime transpiration. While we could not confirm that *MEX1* is the causal gene, we found that stomatal opening was impaired in the *Arabidopsis mex1* mutant, both during light-induced opening at dawn and endogenous reopening at night. Moreover, the grapevine panel was phenotyped in a chamber with low irradiance compared to the outdoor conditions where the leaves had grown, and the most severe phenotype in *mex1* was precisely observed under low light. Thus, *MEX1* is a strong candidate to explain the genetic variation in diel transpiration in grapevine. Nonetheless, we noted that the diel kinetics of the *Arabidopsis* mutant did not resemble any of those observed at the different alleles of the SNP in grapevine. However, given the severity of the *mex1* mutant with a strong impact on growth (Niittylä et al., 2004), it is not plausible that the *MEX1* gene is fully eliminated in a subset of grapevine cultivars. So if *MEX1* is actually the causal gene in grapevine, we speculate that the related genetic variation involves a fine regulation of the activity of the transporter.

An elusive spatiotemporal model for the action of *MEX1* on stomatal opening

How a defect in *MEX1* translates into alterations of stomatal opening remains puzzling. While various starch mutants have different effects on transpiration, our results show that the action of *MEX1* is at least partly independent of starch metabolism. Indeed, neither *pgm* that cannot synthesize starch nor *sex1* that cannot break it down could reproduce the severe phenotype of *mex1* under low light. Consequently, we were unable to conclude whether the processes involved are cell-autonomous: while the *amy3 bam1* mutant clearly showed that guard-cell starch degradation is not required for stomatal opening under white light and during the night, the *pgi1* mutant, which is essentially devoid of starch in the mesophyll cells but metabolizes it in the guard cells, was only marginally affected in diel transpiration. Although it is established that mesophyll-derived glucose uptake is necessary for red light-induced stomatal opening (Flütsch et al., 2020a), this explanation could not account for the reduced rate of stomatal opening at dawn in the starch mutants as they possess massive amounts of soluble sugars in the daytime (Streb and Zeeman, 2012). It is possible, however, that the cocktail of sugars present in the apoplast of these mutants alters sugar homeostasis, which would inhibit stomatal opening according to an unknown mechanism. In line with this hypothesis, contradictory results have been reported about the role of sugars on stomatal movement (Daloso et al., 2016a; Granot and Kelly, 2019), which may be due to the identity of the sugar molecule (Dittrich and Mayer, 1978) or to an interaction with the light intensity (Lugassi et al., 2015). This mesophyll-cocktail model is hard to reconcile with the peculiar phenotype of *mex1* under low light, yet. A different scenario is that maltose excess in *mex1* could trigger autophagy-like chloroplast degradation (Stettler et al., 2009), which may affect the connection between the mesophyll and the stomata that is thought to involve messengers from photosynthetic chloroplasts (Busch, 2014; Lawson et al., 2014; Lawson and Matthews, 2020). Alternatively, chloroplast degradation in *mex1* could also occur directly in the guard cells and disturb their function (Azoulay-Shemer et al., 2015).

Another intriguing finding is that the guard cells of *mex1* are mainly devoid of starch, contrary to its mesophyll cells that over-accumulate it. This observation could be compatible with the idea that the mesophyll of *mex1* cannot provide enough sugars for guard cell starch synthesis (Flütsch et al., 2020a), but one would have to assume that such a sugar deficiency does not affect *sex1*, which is also severely impaired in starch degradation yet accumulates starch in its guard cells. An alternative interpretation could be that MEX1 interacts with GPT to import Glc6P from the cytosol in the guard cells. Supporting this hypothesis, MEX1 has been shown to transport not only maltose but also glucose and Glc1P (Findinier et al., 2017). The Glc6P route may be the most important route for starch synthesis in the guard cells, since fructose 1,6-bisphosphatase activity, which connects starch metabolism with the Calvin-Benson cycle through the production of Fru6P, is low in the guard cells (Hedrich et al., 1985) – yet there is no consensus on this point (Shimazaki et al., 1989; Robaina-Estévez et al., 2017). Moreover, the guard cells of *stp1 stp4*, which cannot uptake glucose due to impaired hexose/proton symporters, have a starch-less phenotype, supporting the idea that guard cell photosynthesis does not support cell-autonomous starch synthesis (Flütsch et al., 2020a). Thus, MEX1 could contribute to guard-cell starch synthesis by importing Glc6P (but not Glc1P otherwise *pgm* would have starch in its guard cells). If true, the transport activity of MEX1 could regulate the sugar homeostasis within the cytosol through the sucrose ‘futile’ cycle (Robaina-Estévez et al., 2017) or the energy transfer from the chloroplast to the cytosol through the ‘shuttle’ system of phosphoglycerate/dihydroxyacetone phosphate (Shimazaki et al., 1989; Shimazaki et al., 2007), which are thought to be of utmost importance for light-induced stomatal movements.

Ultimately, the metabolic activity supported by MEX1 is likely to promote the activity of AHA1, the major H⁺-ATPase in the guard cells that energizes solute transport and impacts both daytime and nighttime transpiration (Merlot et al., 2007; Blatt et al., 2014; Costa et al., 2015). Notably, H⁺-ATPase activity in the guard cells stimulates the activity of inward K⁺ channels, which are key effectors in stomatal opening. The *kinless* mutant, which is impaired in inward K⁺ current, displays the same phenotypes as *mex1*, with reduced rate of stomatal opening at dawn and reduced nighttime reopening (Lebaudy et al., 2008). Thus, the MEX1-induced metabolic configuration could promote stomatal opening by enhancing the import of K⁺ in the guard cells through the stimulation of AHA1 activity. Interestingly, the guard cells of the *aha1* mutant are impaired in starch degradation and stomatal opening upon blue light, yet the mechanistic connection between starch metabolism and H⁺-ATPase activity remains unexplained (Blatt, 2016; Horrer et al., 2016). Moreover, *aha1* guard cells also show defective starch synthesis and impaired stomatal opening upon red light (Flütsch et al., 2020b). It is thought that AHA1 energizes the uptake of mesophyll-derived sucrose, which generates turgor in the guard cells and concomitantly supports starch biosynthesis therein, further supporting the idea that stomatal response to red light is disconnected from guard cell starch metabolism (Flütsch et al., 2020b).

Daytime control of nighttime stomatal reopening

Since the first observation one century ago that stomata reopen progressively during the night (Loftfield, 1921), this behaviour has been reported in many species (Schwabe, 1952; Caird et al., 2007). While it has no clearly established function in plants (Resco de Dios et al., 2019), the resulting nighttime transpiration lowers water use efficiency (Coupel-Ledru et al., 2016). In *Vicia faba*, nighttime reopening is enhanced when photosynthesis is increased in the previous day (Easlon and Richards, 2009), leading to the suggestion that the photosynthetic activity is ‘memorized’ during the day to instruct stomatal movements in the following night. Our results are broadly consistent with this view, yet demonstrate that starch turnover in the mesophyll cells cannot solely account for such a memory. Understanding the role of MEX1 in this process would allow to disconnect guard cell behaviour from leaf starch

metabolism, thus opening the way for reducing nighttime transpiration without altering photosynthesis in the daytime and carbon status at night. It therefore appears as a promising strategy to breed crops for more efficient use of water.

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Supplementary

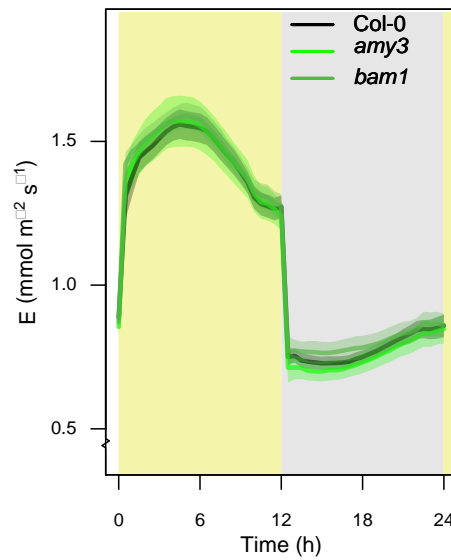


Figure S1. Diel transpiration dynamics of single mutants affected in guard cell starch metabolism. A,B. Diel transpiration dynamics of the wild-type Col-0 compared to *amy3* (A) and *bam1* (B). The 95% confidence interval is shown ($6 \leq n \leq 8$). These data correspond to the average of the first two control days presented on Figure 4 and S5.

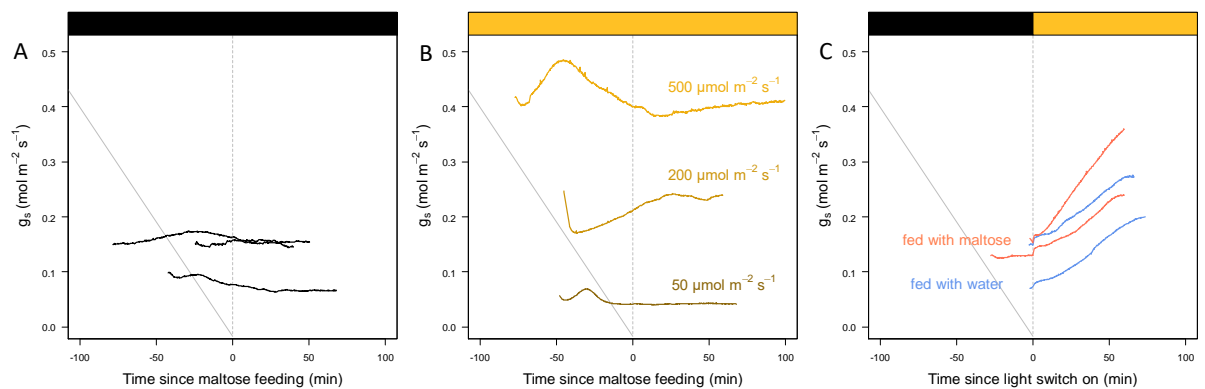


Figure S2. Feeding maltose through the transpiration stream does not affect stomatal conductance. Gas exchange experiments on detached leaves of Col-0 that were xylem-fed with water or 1 mM maltose. A. Maltose-feeding in darkness at the end of the night. B. Maltose-feeding in the light (50, 200

or $500 \mu\text{mol m}^{-2} \text{s}^{-1}$) during the daytime. C. Leaves fed with water or maltose at the end of the night, subjected to extended darkness and then to light ($500 \mu\text{mol m}^{-2} \text{s}^{-1}$).

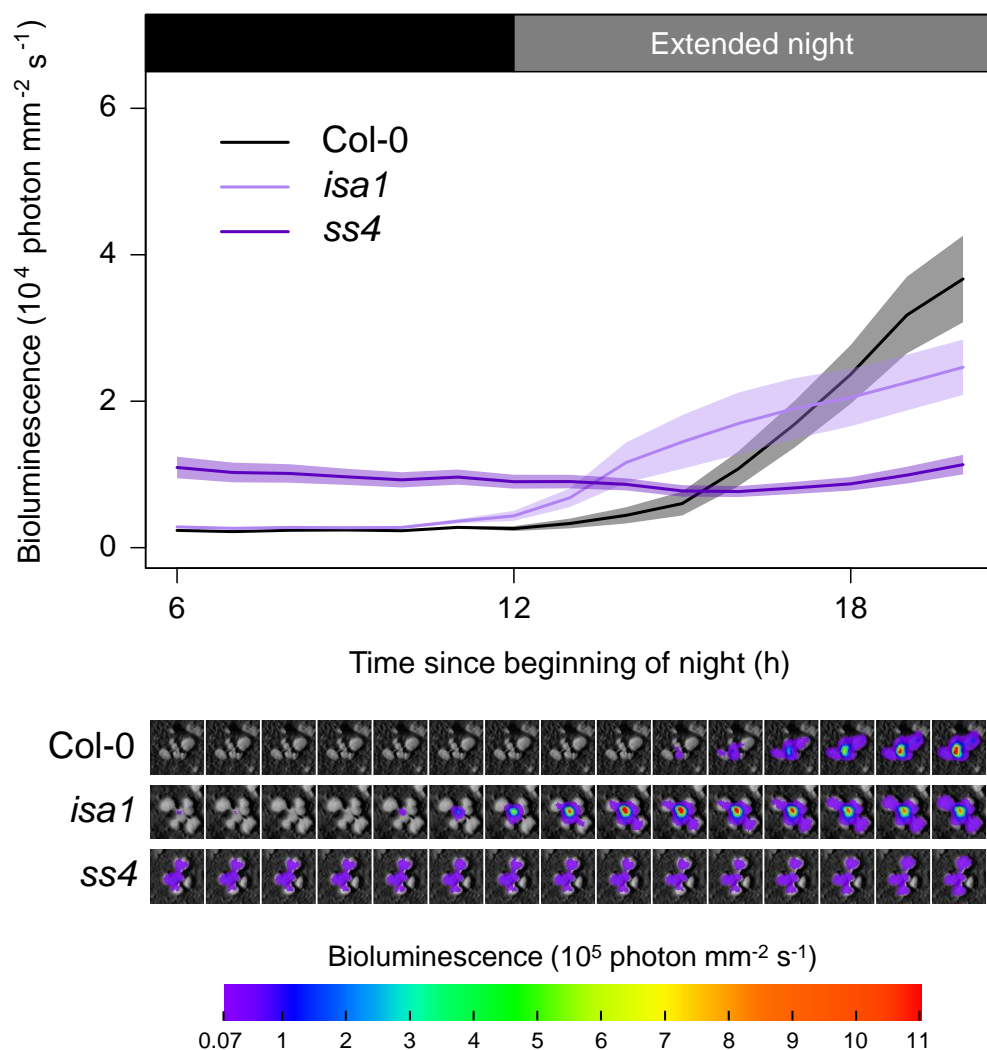


Figure S3. Starvation dynamics in contrasting starch mutants. A starvation luminescent reporter was inserted into Col-0, *ss4* and *isa1*. Plants were grown during two weeks in soil at a 12-h photoperiod and assayed for bioluminescence from the middle of the night to an eight-hour prolongation of the night. The black and grey rectangles represent the expected end of night and the eight-hour prolongation of the night, respectively. Error bars are mean \pm s.e.m. ($n = 24$).

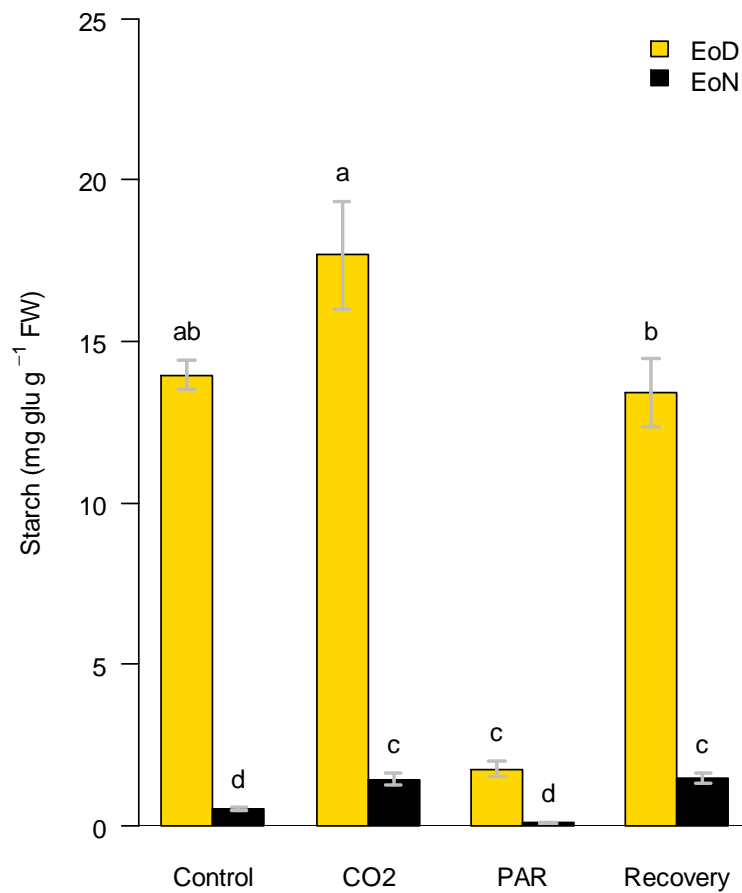


Figure S4. Effects of elevated CO₂ and low light on the starch content of the wild-type. Leaf starch content determined at the end of the day and night periods for Col-0 subjected to the environmental scenario described in Figure 4. The control and recovery correspond to the first control and recovery day, respectively. Error bars are means \pm SE ($n = 4$). Letters denote significant differences after a Kruskal-Wallis test ($\alpha = 5\%$) followed by multiple comparison of mean ranks.

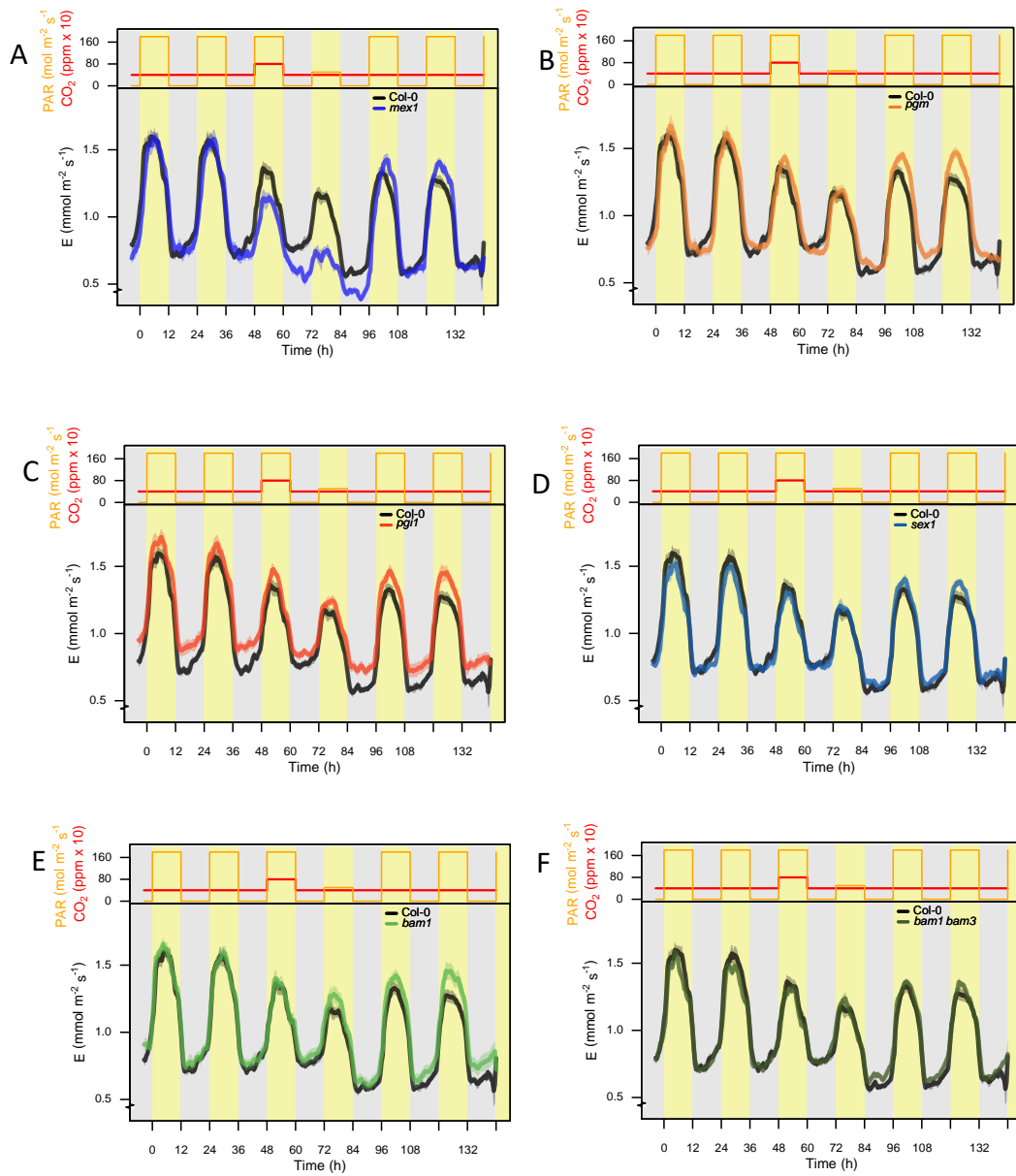


Figure S5. Detail of the effects of elevated CO₂ and low light on the diel transpiration dynamics. Individual kinetics of the genotypes presented on the heatmap in Figure 4, as compared to their wild-type.

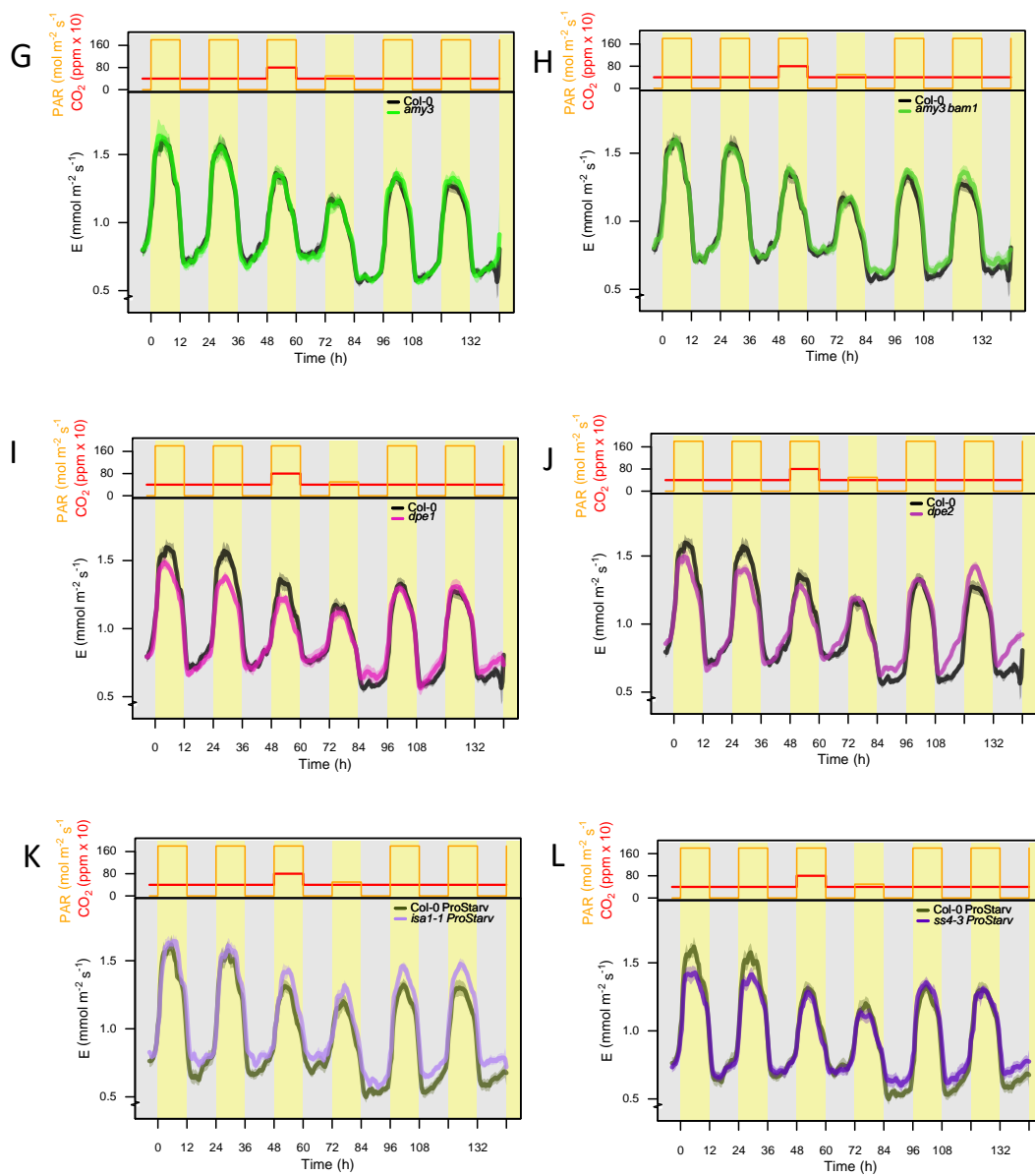


Figure S5. Continued.

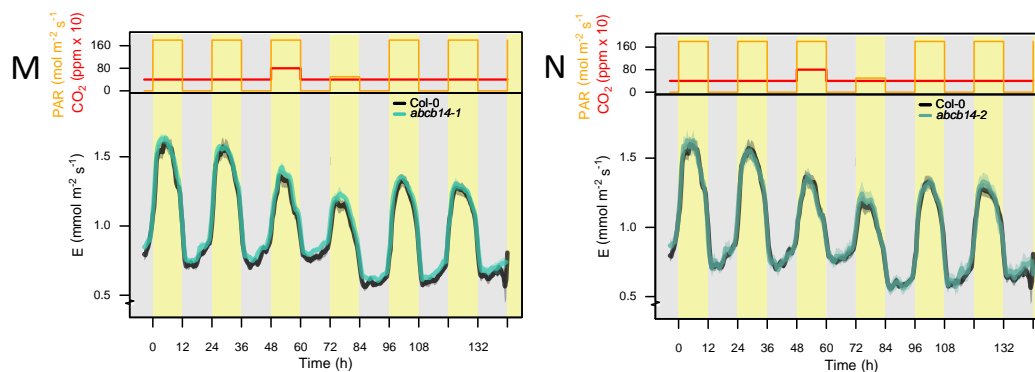


Figure S5. Continued.

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Chapter 4

Genetic control of nighttime transpiration through MYB-specified epidermal sophistication in grapevine

Outline

This chapter focuses on the GWAS result for nighttime transpiration on detached leaves of grapevine. The genes underlying the most significant genomic region were investigated and pointed towards the involvement of epidermal structures, other than stomata, modulating nighttime transpiration. By using online expression databases for grapevine, we identified a gene in the region that may control several aspects of epidermal development and nighttime transpiration.

Contributors

This chapter was based on research I conceptualized with my supervisors Florent Pantin¹ and Thierry Simonneau¹. For data collection, I was helped by Lucille Roux¹ (grapevine GWAS experiment), the postdoctoral researcher Aude Coupel-Ledru¹ (grapevine GWAS experiment and QTL analysis SyrahxGrenache), Pilar Gago⁴ (phenotyping of trichome density in the SyrahxGrenache population) and Myriam Dauzat¹ (Phenopsis monitoring). I have performed all data analyses, after initial advices on grapevine quantitative genetics by Aude Coupel-Ledru¹, Timothée Flutre² and Agnès Doligez³. I performed the candidate gene analysis advised by Patrice This³, Vincent Segura³ and Timothée Flutre². I wrote the first draft corrected by Florent Pantin¹.

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Abstract

Plants transpire and are vulnerable to drought stress. Breeding crops for reduced nighttime transpiration would allow saving water without penalizing photosynthesis. Here we show that nighttime water loss in grapevine is congruent with leaf epidermal morphogenesis, likely controlled by the MYBF2 transcription factor. Analyzing nighttime transpiration in a grapevine association panel revealed a main locus that co-localized with an association for leaf hairs and nanoridges on the epidermal cuticle. Further analyses supported a model whereby the underlying candidate MYBF2 specifies the development of both prostrate trichomes and cuticular nanoridges, with the latter mirroring deeper changes in the cuticle that enhance water loss. MYBF2 belongs to a subfamily of R2R3-MYBs normally involved in secondary metabolism, also responsible for the pink colour in tomato. We propose that neofunctionalization of MYBF2 has been selected during grapevine domestication for the production of trichomes—a barrier against pathogens, while embarking morphogenesis of cuticular nanoridges and enhancement of nighttime water loss as a side-effect.

Introduction

Tissue morphogenesis creates patterns of cellular structures with dedicated functions in their prevailing environment. At the interface with the atmosphere, the epidermis of plant leaves is made of unspecialized pavement cells that are interspersed with two-celled valves called stomata, and outgrown hairs called trichomes (Larkin et al., 1997). To accommodate the dry atmosphere when colonizing the land 500 million years ago, plants have evolved a hydrophobic layer of cuticle made of cutin and waxes that covers the epidermal cells, acting as a barrier against water loss (Kerstiens, 2006; Kong et al., 2020; Riederer and Schreiber, 2001). The composition and morphology of the leaf cuticle are extremely variable between and within species (Fernández et al., 2017; Ickert-Bond et al., 2018; Konlechner and Sauer, 2016), yet it remains elusive to what extent changes in the properties of the cuticle affect the waterproof capacity of the epidermis. Punctuating this barrier, stomata have evolved for optimal control of gas exchange. These adjustable pores open to facilitate the capture of atmospheric CO₂ for photosynthesis, and close to avoid excessive water loss –transpiration– depending on environmental conditions (Harris et al., 2020; Hetherington and Woodward, 2003). Trichomes show extensive variation in type and abundance, and may provide protection against predators and pathogens, as well as mitigation of leaf microclimate and gas exchange (Bickford, 2016; Johnson, 1975). Thus, the leaf epidermis illustrates how tissue development can be evolutionary shaped to integrate an array of external contingencies and selective pressures.

Among the multitude of stresses that plants face, drought is a major concern that challenges crops and forests increasingly with climate change (Brodribb et al., 2020; IPCC, 2014; Lesk et al., 2016). Since open stomata are the main route for water loss, they are a favoured target for breeding plants with enhanced drought tolerance (Gupta et al., 2020). However, closing stomata to save water often results in lowering carbon assimilation and yield, making this approach agriculturally inefficient in many environmental scenarios (Tardieu et al., 2018). Thus, different strategies are being developed to enhance the water use efficiency, namely the carbon fixed per unit of transpired water (Condon et al., 2004). For instance, engineering a key ion channel to boost the solute fluxes that drive stomatal movements allows stomata to better track changes in light conditions: these “enhanced” stomata open faster upon illumination, thus relieving the CO₂-limitation on photosynthesis, and close more rapidly upon darkness, thereby reducing the time stomata lose water when light is insufficient to fuel photosynthesis (Papanatsiou et al., 2019). A further strategy to enhance water use efficiency is to reduce nighttime transpiration (Coupel-Ledru et al., 2016). This ‘unproductive’ water loss can reach up to 30% of daytime transpiration (Caird et al., 2007). Nighttime transpiration is thought to originate mainly from incomplete stomatal closure, and to a lesser extent from residual permeability of the cuticle (Caird et al., 2007). However, the molecular determinants of nighttime transpiration remain largely unknown (Costa et al., 2015; Isner et al., 2017).

Here, we analysed the genetics of nighttime transpiration in grapevine, a crop species threatened by climate change (Fraga et al., 2016) that could yet benefit from a better use of its large genetic diversity (Morales-Castilla et al., 2020; Wolkovich et al., 2018). We first developed a high-throughput pipeline for monitoring the transpiration of detached leaves, and phenotyped a grapevine association panel (Flutre et al., 2020; Nicolas et al., 2016). Our genome-wide association study (GWAS) revealed a main association for nighttime transpiration. Examination of the neighbouring genes revealed no obvious candidate relevant to stomata, but pointed to other facets of epidermal development. Accordingly, a strong association was found at the same locus for leaf hairs, called prostrate trichomes, and for cuticular nanoridges, called striae. The allele that conferred enhanced nighttime transpiration also conferred higher abundance of both trichomes and striae. Genetic and physiological analyses discarded a direct role for leaf hairs in enhancing water loss, and suggested that cuticular nanoridges

mirrored other changes in the cuticle composition or morphology that originate higher epidermal permeability. Co-expression supported a model whereby the candidate MYBF2 controls the morphogenesis of both prostrate trichomes and nanoridges. MYBF2 belongs to a subfamily of R2R3-MYB transcription factors that is usually involved in flavonol metabolism (Czemmel et al., 2009; Stracke et al., 2007; Zhang et al., 2015). We propose that neofunctionalization of MYBF2 has been retained during grapevine domestication as a provider of trichomes that act a barrier against pathogens (Kortekamp and Zyprian, 1999), yet at the expense of nighttime water loss through cuticular changes mirrored by nanoridges. Our findings pave the way for breeding plants with enhanced resilience to pathogens and climate change, through a framework that integrates different facets of epidermal morphogenesis.

Material and methods

GWAS for nighttime transpiration and epidermal structures

Plant material and experimental design

Plants for 279 cultivars of a grapevine diversity panel (Nicolas et al., 2016) were generated from cuttings obtained from the Vassal-Montpellier Grapevine Biological Resource Center (Marseillan-Plage, France). Non-grafted plants were grown outdoors in individual 3L pots with a mixture containing 30:70 (v/v) loamy soil and organic compost in the nursery at the Pierre Galet experimental vineyard of INRAE-SupAgro (Montpellier, France). Ten plants per cultivar were pruned to produce a single shoot, cut at a height of 3 meters and ferti-irrigated to a well-watered regime. Young flowers were systematically removed to avoid differences in vegetative and reproductive stages between genotypes. The experiment was carried out on 2-years-old plants during summer 2019, from mid-June to mid-July. Fully developed leaves located at the 8th-12th phytomere were collected in the morning. Immediately after cutting, leaves were immersed in distilled water and their petioles were recut to avoid cavitation. Leaves were placed in tubes containing artificial xylem sap (AXS) containing 2 mol m⁻³ KH₂PO₄, 1 mol m⁻³ MES and 0.4 mol m⁻³ Ca(NO₃)₂ (Coupel-Ledru et al., 2016). Petioles were tightly sealed to the container cap to prevent direct evaporation of AXS. Every day, 70 leaves of random selected genotypes were collected in the midmorning and installed before midday in the PHENOPSIS automaton (Granier et al., 2006, https://www6.montpellier.inrae.fr/lepe_eng/M3P). This is a growth-chamber initially designed for the cultivation and monitoring of *Arabidopsis thaliana*. PHENOPSIS was used to expose grapevine leaves to stable daytime conditions for VPD (1.5 kPa), air temperature (25 °C) and light (180 μmol photons m⁻² s⁻¹) for nine hours. Doing that, we circumvented that daytime conditions influenced nighttime transpiration, as reported before (Easlon and Richards, 2009). Then, 10 night-hours were simulated by switching lights off, closely following the outdoor day/night cycle. Air temperature (25 °C) and VPD (1.5 kPa) stayed constant at night while leaves' weight losses were monitored every 30 minutes with a mobile Precisa XB 620M scale (Precisa Instruments AG, Dietikon, Switzerland). In the following morning, leaves were taken off the platform for epidermal structure and leaf surface analysis and replaced by a new batch of 70 leaves. This protocol was executed during one month in order to obtain six replicates from 5-6 plants per genotype.

Nighttime transpiration was estimated from gravimetical data of PHENOPSIS using R (R Core Team, 2019). First, data was cleaned for potential weighing errors. These errors could arise from unstable conditions during measurement as turbulent air or touchy neighbouring leaves. Aberrant points were automatically detected based on biological limits and regression influence diagnostics, and checked manually. Then, nighttime transpiration was estimated by a linear model fitted on individual leaves'

weight losses at night. The slope was divided by leaf area to obtain the transpiration per leaf area unit. Leaf area was determined by digitizing individual leaves with a flatbed scanner (Epson 4990, Seiko Epson Corp., Nagano, Japan). Pixels corresponding to leaf area were segmented from the background by three threshold algorithms using Python (van Rossum and Drake, 2003). First, an RGB threshold selected green pixels. Then, a threshold was applied based on the ratio of the green channel to the red and blue channel. Afterwards a HSV threshold was applied with help of the matplotlib library (Hunter, 2007)). Finally, leaf or plant contours are drawn with OpenCV libraries (Bradski, 2000) and a final mask is created by including all the pixels within the contour higher than a minimum pixel value. Next, nighttime transpiration was corrected for average VPD conditions in PHENOPSIS. Around 4% of the total number of leaves were discarded from further analysis because of cavitation doubts.

Quantification of epidermal structures and nighttime transpiration

Epidermal structures were studied on imprints obtained on each leaves' abaxial side. Quick-drying nail polish (Maybelline, New York, NY, USA) was applied on the area between the major and lateral vein at the lower side of the leaf (Gago et al., 2019). When necessary, leaf hairs were removed by adhesive tape before the application of nail polish. The dried imprint was pulled off by adhesive tape and fixed on a microscope slide. Not all nail polish imprints could be pulled off correctly, requiring the application of resin for a silicone leaf impression (President Dental GmbH, Allershausen, Germany) prior to nail polish application. Impressions were then viewed with a 10X magnification on a fluorescence Leitz DMRB microscope (Leica GmbH, Wetzlar, Germany) equipped with a Manta G201B camera (Allied Vision Technologies, Stadtroda, Germany). Images were captured with μ Manager software (Edelstein et al., 2014). The level of cuticular striae was manually scored within a 0.6 mm² area as follows: 0 (absent), 1 (around guard cells), 2 (strongly present around guard cells), 3 (tissue-wide striae) or 4 (strong tissue-wide striae).

The density of erect and prostrate trichomes in between the main veins was notated according to the classification of the Organisation Internationale de la Vigne et du Vin (Organisation Internationale de la Vigne et du Vin, 2009, indices OIV 084 and 085). A single score from 1 (low) to 9 (high) was attributed to each genotype based on visual inspection of several leaves' abaxial side with an eyepiece.

Statistical modelling of phenotypic data

Exploratory data analysis was performed to check for data normality (Shapiro-Wilk test, Royston, 1992). When the data was skewed, phenotypic data was transformed by natural logarithm. Single effects of leaf orientation in the nursery, position in chamber and day of measurement were tested by regression analysis, t-tests and one-way analysis of variance (ANOVA). Spatial heterogeneity for transpiration in PHENOPSIS was analysed by kriging. Iterative kriging was performed to increase the precision of non-random spatial variation (Zas, 2006). Best Linear Unbiased Predictors (BLUPs) of genetic values were then estimated using univariate linear mixed models. Models tested were fitted by maximum likelihood (ML) and included a random genotypic effect (Bates et al., 2015), completed or not by fixed effects as among those cited above. The best model was chosen based on the Akaike information criterion (AIC, Burnham and Anderson, 2004). Assumptions, such as homoscedasticity and normality were checked visually by looking at residuals and best linear unbiased predictors (BLUPs) of genotypic values. The broad-sense heritability (H^2) was calculated as the ratio between the genotypic variance and total variance divided by the average number of replicates.

Genome Wide Association Study

A genome-wide association study (GWAS) was conducted to identify associations between BLUPs of the association panel and 63,037 SNPs (with minor allele frequency, MAF < 0.05). SNP data were based

on microarray and genotyping-by-sequencing (GBS) data followed by imputation (Flutre et al., 2020). The level of relatedness (kinship) between individuals was estimated based on SNPs with a linkage disequilibrium (LD) threshold of 0.5 using the kinship estimator of VanRaden (Flutre et al., 2020; VanRaden, 2008). The weak population structure as described in Nicolas *et al.* (Nicolas et al., 2016) was ignored. Quantitative trait loci (QTL) were detected using a SNP-by-SNP model implemented in GEMMA version 0.97 (Zhou and Stephens, 2012). A linear mixed model was used to test for every SNP: $BLUP(g) \sim 1\mu + M_{a,p}\beta_p + u + \varepsilon$, where $M_{a,p}$ is a vector with genotypes at the p_{th} SNP and $\varepsilon \sim N_N(0, \sigma_e Id)$ with N the normal distribution of dimension N , 0 a vector of zeros and Id the identity matrix of dimension $N \times N$. Relatedness was controlled by a random effect u , based on additive genetic relationships in a covariance matrix. The effect of SNPs was significant when the p value from the Wald test statistic was smaller than the Bonferroni threshold.

Transpiration from leaf to whole-plant

Plant material and experimental design

Three different methods to assess nighttime transpiration were compared in four genotypes of the association panel. The same biological material was used as described above. Five detached leaves per genotype were collected in the nursery in July 2019. Cut leaves were directly immersed in water and recut to avoid cavitation. Leaves were put in a tube with AXS and were installed in PHENOPSIS together with two well-watered plants of the same genotype. A leaf of one of the plants was placed in a leaf cuvette of a Li-Cor 6400 gas analyser system (Li-Cor Inc., Lincoln, NE, USA) connected to a dew-point generator to control humidity (Li-Cor 610). The environmental conditions in the Li-cor as well as in the climate chamber were similar (VPD 1.5 kPa, air temperature 25 °C) including the 10-hours night cycle closely following the outdoors day/night cycle. Weight losses of detached leaves were monitored by the PHENOPSIS automaton as described before. Whole-plant weight losses were continuously measured on a scale (OHAUS Defender 5000, Parsippany, NJ, USA), connected to a data logger. Evaporation of the soil was avoided by putting a plastic bag around the pot.

Transpiration analysis

First, gravimetrical data of detached leaves and whole-plants was cleaned for potential weighing errors. These errors could arise from unstable conditions during measurement as turbulent air or touchy neighbouring leaves. Aberrant points were automatically detected and checked manually. Then, nighttime transpiration was estimated by a linear model fitted on the leaves or plant weight losses at night. The slope was divided by leaf area to obtain the transpiration per square meter. Leaf area was determined by digitizing individual leaves with a flatbed scanner (Epson 4990, Seiko Epson Corp., Nagano, Japan). Whole-plant leaf area was estimated using an allometric relationship per genotype between secondary vein length and leaf surface. Transpiration values were corrected for VPD conditions and Pearson's correlation coefficients were calculated between transpiration values of detached leaves, whole-plant and by the gas-exchange analyser.

QTL analysis for prostrate trichomes in a biparental population

Plant material and experimental design

Trichomes density was also assessed on a grapevine mapping population ($S \times G$) previously described (Adam-Blondon et al., 2004; Huang et al., 2012). It consisted of a pseudo-F1 progeny of 191 individuals from a cross between two cultivars, Syrah and Grenache, and was maintained at Montpellier SupAgro Domaine du Chapitre (Hérault, France). The $S \times G$ population was planted in two blocks. Each individual from the progeny was planted in two elementary plots (one per block) comprising five plants each.

Quantification of trichomes

The density of erect (OIV085) and prostrate (OIV084) trichomes was scored in 2011 according to the classification of the Organisation Internationale de la Vigne et du Vin (Organisation Internationale de la Vigne et du Vin, 2009). A single score from 1 (low) to 9 (high) was attributed to each of the five plants per genotype in the well-watered block, based on visual inspection of several leaves' abaxial side with an eyepiece, and a genotypic mean was then calculated.

QTL analysis

QTL detection was then performed using a framework linkage map of 153 simple sequence repeat (SSR) markers (Huang et al., 2012). QTL detection was carried out on genotypic means with R/qtl package (Broman et al., 2003) using the consensus map which combined information from the two parents. Multiple QTL regression was carried out with the "stepwiseqtl" function following Huang et al. (2012), using a genome-wide error rate of 0.05.

Analysis of nighttime transpiration in *Arabidopsis thaliana*

Plant material, growing conditions and experimental design

Arabidopsis ecotypes Col-0 and Col-5 were grown in 30:70 (v/v) loamy soil and organic compost in PHENOPSIS at a 12-hours photoperiod ($180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Air temperature and VPD were 21 °C and 0.8, respectively, during day and night. Each pot was weighed twice a day and re-watered to its well-watered level ($1.35 \text{ g}_{\text{water}} \text{ g}^{-1} \text{ dry soil}$). One week before bolting stage, the water losses of plants were measured gravimetrically in PHENOPSIS by the protocol described before. The soil was covered by a layer of cellophane to avoid evaporation of the soil. Plants water losses were monitored of 5 replicates of each genotype during 6 consecutive days. Plants were manually re-watered if soil water content was lower than its well-watered level. Plants were imaged at least once a day.

Transpiration analysis

Nighttime transpiration was estimated from gravimetrical data of PHENOPSIS using the protocol described before. Transpiration was corrected for rosette area obtained from topview images in PHENOPSIS and analysed with Python as described before for grapevine leaf area scans. Then, the transpiration was corrected for the VPD conditions at night in the chamber. The average nighttime transpiration of each plant was calculated based on six consecutive nights.

Hair removal effect on nighttime transpiration in grapevine

Plant material and experimental design

Leaves were collected in June and July 2020 from 20-years old vines in the Pierre Galet experimental vineyard located at the INRAE-SupAgro campus (Montpellier, France). Varieties were grafted on the rootstock Selection Oppenheim No. 4 (SO4) and grown in an artificial soil of sand and small stones completely depending on ferti-irrigation. The effect of prostrate trichome removal on transpiration was tested in leaves of Syrah, Lledoner Pelut, Macabeu, Pinot Noir and Pinot Meunier. Prostrate trichomes were removed from the leaf lamina by gently hand rubbing and stayed 1, 3 or 14 days attached to the plant. A similar mock treatment was performed on the leaves of glabrous Grenache noir. Leaves were collected in the morning following the same protocol as above. Then, leaves were assigned to tubes containing AXS or AXS enriched with $500 \mu\text{M} \pm$ abscisic acid (Sigma-Aldrich, St. Louis, MO, USA) and installed in PHENOPSIS. Each treatment contained five replicates obtained of at least four plants.

Statistical analysis of nighttime transpiration

Nighttime transpiration was estimated from gravimetric data of PHENOPSIS using the protocol above. Transpiration was corrected by scanned leaf area (protocol described before) and the average VPD at night in the chamber.

Stomatal responses of striaed and striae-free leaves

Plant material and experimental design

Leaves were collected from 20-years old vines in the Pierre Galet experimental vineyard located at the INRAE-SupAgro campus (Montpellier, France). Four leaves per genotype were collected of at least three plants in August 2020. Leaves for stomatal response analysis were cut in the evening prior to the experiment, immersed in water and recut to avoid cavitation. Leaves were put in tubes filled with AXS and allowed to rehydrate in the dark at room temperature. The following day, leaf gas exchange was measured in a leaf cuvette of a Li-Cor 6400 gas analyser system (Li-Cor Inc., Lincoln, NE, USA) connected to a dew-point generator to control humidity (Li-Cor 610, Li-Cor Inc., Lincoln, NE, USA). After a short acclimatization period in darkness (air temperature 25 °C, VPD 2 kPa, flow rate 500 mol s⁻¹, CO₂ 400 ppm), light was switched on to analyse stomatal response to light (500 μmol photons m⁻² s⁻¹). After two hours, light was switched off again to analyse stomatal response to darkness.

Results

Co-localization of an SNP marker for nighttime transpiration and trichomes. To investigate the genetics of nighttime transpiration in grapevine, we developed a high-throughput screening method in controlled conditions on detached leaves. In a grapevine canopy, the spatiotemporal variability in leaf microclimate and the co-occurrence of several leaf developmental stages make transpiration highly heterogeneous (Albasha et al., 2019). We reasoned that genuine genotypic values could be better captured using single, fully expanded leaves collected from plants grown outdoors, placed in a chamber with controlled microclimate and fed with artificial xylem sap (Coupel-Ledru et al., 2017). Transpiration was monitored gravimetrically by diverting an existing phenotyping automaton developed for Arabidopsis (Granier et al., 2006). Preliminary experiments indicated that the transpiration of grapevine detached leaves could respond to day/night regimes during at least 48 h (Chapter 2). Using this system, similar nighttime transpiration rates were obtained when compared to an attached leaf monitored with an infrared gas analyser or to a whole-plant placed in the same chamber at the same time (Chapter 2).

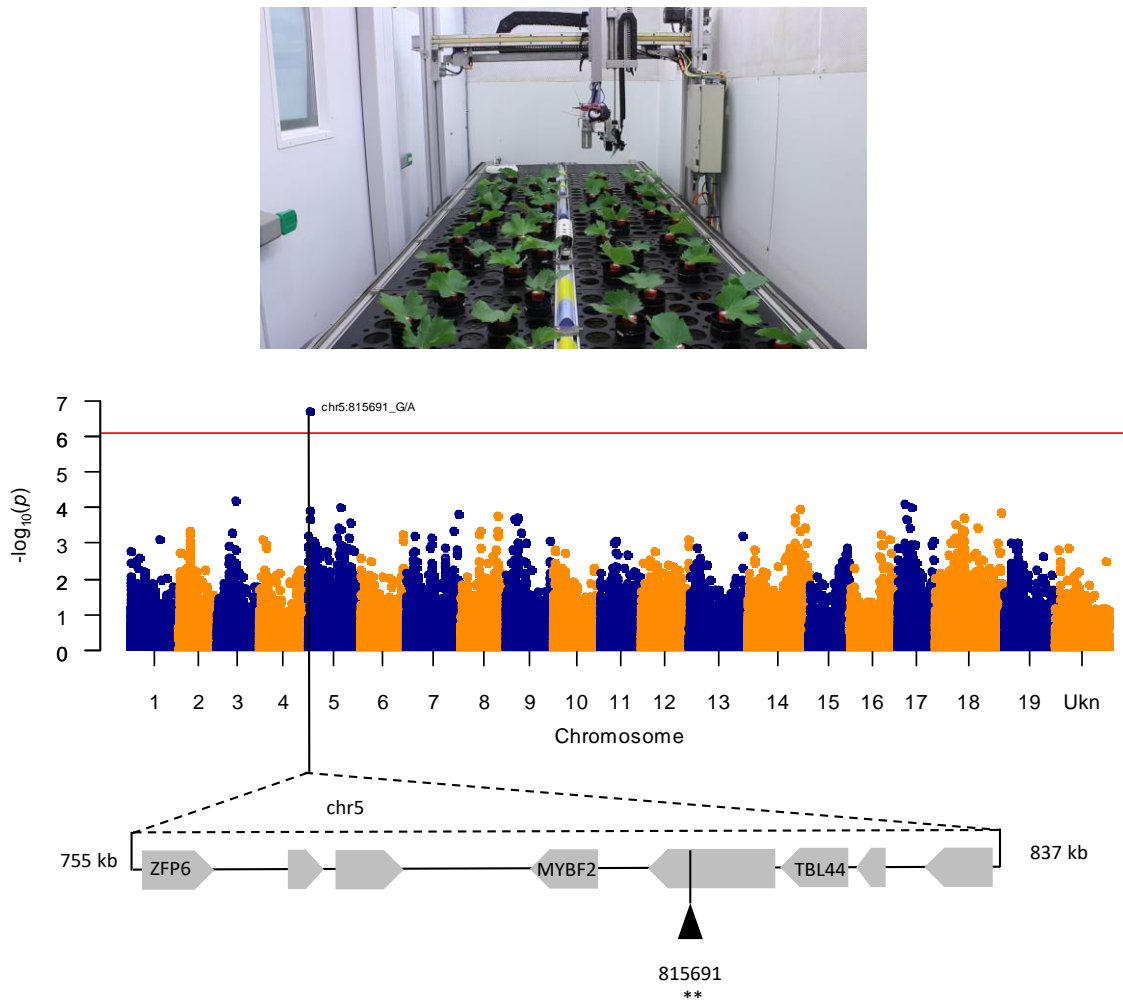


Figure 1: GWAS results for nighttime transpiration on detached leaves in a grapevine association panel. Leaves were installed in the robot before midday and monitored for 24-h. The red line denotes the Bonferroni threshold. Three genes in the ~100 kb region surrounding the SNP were designated for their possible role in nighttime transpiration.

We then applied this protocol on a grapevine association panel (Nicolas et al., 2016). Genotypic values for nighttime transpiration ranged from 0.028 to 0.067 $\text{mmol m}^{-2} \text{s}^{-1} \text{kPa}^{-1}$, with a broadsense heritability of 0.30. We conducted a genome-wide association study (GWAS) and found a strong marker association on chromosome 5 (Fig. 1A). This marker explained 15% of variation in nighttime transpiration. This result contrasts with a previous genetic analysis in an F1 population between two grapevine cultivars, Syrah and Grenache, which identified several quantitative trait loci (QTL) for nighttime transpiration but none on chromosome 5 (Coupel-Ledru et al., 2016). This may be explained by differences in the genetic material (association panel vs. biparental population), growth conditions (outdoors vs. greenhouse), and/or experimental setup (detached leaves vs. whole plants). We then looked at the list of candidate genes in the genomic region around the detected SNP. We could not identify any obvious candidate relevant to stomata, even when the list was extended from 100 kb to a 500 kb region spanning the SNP on both sides (Supplementary Table 1). However, within a region of 30 kb around the SNP, we noticed several genes involved in, or related to, epidermal patterning in

other species: *ZINC FINGER PROTEIN 6-LIKE (ZFP6)* that encodes a transcription factor involved in the initiation of trichomes in *Arabidopsis* (Zhou et al., 2013); *MYBF2*, a very close paralog of tomato *MYB12*, which is an R2R3-MYB transcription factor that specifies the pigmentation of the fruit surface in tomato (Adato et al., 2009; Ballester et al., 2010; Fernandez-Moreno et al., 2016); and *TBL4*, an uncharacterized member of the *TBL* gene family that contains two genes important for cellulose deposition on trichomes in *Arabidopsis* (Bischoff et al., 2010).

Because we had noticed a large variability in leaf trichomes of the grapevine genotypes, we scored the association panel for related traits. Trichomes are mostly found on the abaxial side of the leaf and two types can be distinguished (Gago et al., 2016): erect (like in *Arabidopsis*) and prostrate (cottony hairs). Performing a GWAS on prostrate trichomes in the abaxial lamina revealed an SNP that co-localized exactly with the SNP on chromosome 5 for nighttime transpiration (Fig. 2). This SNP was not found for erect trichomes (Fig. Suppl. 1). Previous genetic analyses for prostrate hairs in various F1 *Vitis* populations also detected a significant QTL in the region surrounding the SNP on chromosome 5 (Divilov et al., 2017; Kono et al., 2018). We also scored prostrate trichomes in an F1 population between the glabrous Grenache and the pubescent Syrah, two varieties that are not part of the 279-accession panel, and found a major QTL at the same locus (Fig. Suppl. 2). Overall, the perfect SNP co-localization between prostrate trichomes and nighttime transpiration in our study suggested that both traits are mechanistically related by the same gene.

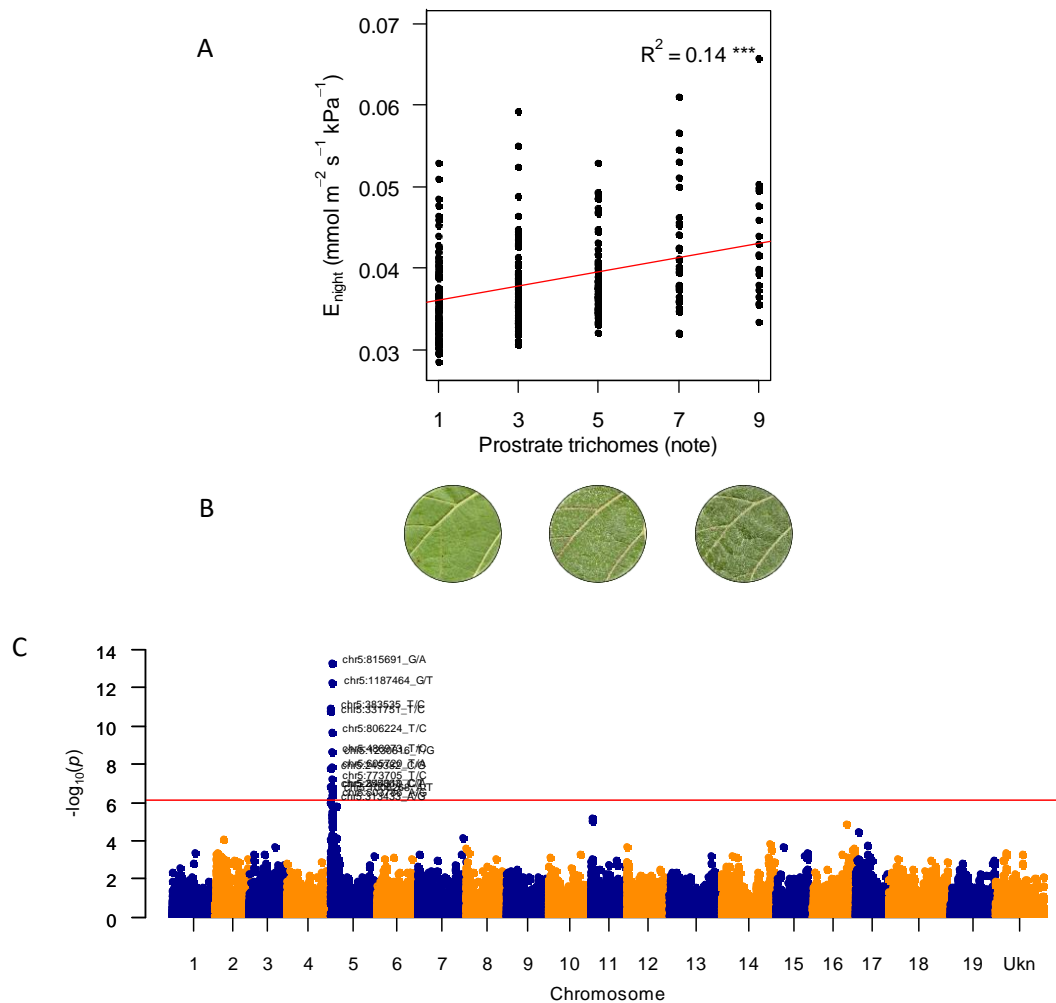


Figure 2: GWAS for prostrate trichome level. A) Correlation between prostrate trichomes and nighttime transpiration on detached leaves. The coefficient of variation was calculated by a linear model (R^2). B) Three examples of different density of prostrate trichomes on the abaxial side of the leaf from no trichomes (1, left) to a dense cover of trichomes (3, 7, middle and right respectively). C) Manhattan plot for the level of prostrate trichomes in a grapevine association panel.

Prostrate trichomes are not functionally related to nighttime transpiration. Leaf trichomes have long been suspected to modulate transpiration rate, either by modifying the leaf microclimate or by augmenting the epidermal surface area subjected to evaporation (Bickford, 2016). However, the complexity of these opposing effects and their possible feedback on stomatal aperture and development (Galdon-Armero et al., 2018) makes it difficult to predict whether the presence of trichomes has a positive or negative effect on transpiration. In our panel, the allele that conferred higher abundance of prostrate trichomes was the one associated with higher nighttime transpiration.

To assess the functional relationship between transpiration and trichomes, we first tested if abolishing trichome morphogenesis results in lower transpiration rate in *Arabidopsis*. We used a glabrous mutant, *gl1-1* (also known as Col-5; Bloomer et al., 2012), which is impaired in the R2R3-MYB transcriptional regulator GLABRA1 that normally promotes trichome initiation (Ishida et al., 2008). Contrary to our

expectations, monitoring the diel (24-h) transpiration rate on whole *Arabidopsis* plants revealed a slight but significant increase in nighttime transpiration in the glabrous mutant (Fig. Suppl. 3). We confirmed this small effect using gas exchange analysis on leaves subjected to extended darkness (Fig. Suppl. 4). When the contribution of stomata to the transpiration flux was lowered by feeding the petiole with the drought hormone abscisic acid (ABA), the difference between Col-0 and *g1-1* vanished (Fig. Suppl. 4). These data suggested there is no strong, direct effect of trichomes on transpiration in *Arabidopsis*. Since trichomes are erect and sparse in this species, we therefore addressed the possibility that a dense layer of prostrate trichomes is necessary to substantially increase water loss in grapevine.

To tackle this question, we first designed pairwise comparisons for diel transpiration using grapevine varieties with contrasting pubescence that were not assayed in the association panel. We compared the glabrous Grenache to the pubescent Syrah – a trichome phenotype that is mostly controlled at the locus identified on chromosome 5 (Fig. Suppl. 2). Notably, nighttime transpiration was more than 43% higher, and this difference was maintained when ABA was added in the feeding solution, suggesting a non-stomatal origin (Fig. 3 A). We then compared the glabrous Grenache to Lledoner pelut, a somatic mutant of Grenache with pubescent leaves (Fernández et al., 2008; This et al., 2006), for which the causal mutation has not been identified. Again, nighttime transpiration was 40% higher in Lledoner Pelut than in Grenache irrespective of the ABA treatment (Fig. 3 A). We also compared the weakly hairy Pinot noir to its tomentose mutant Pinot Meunier, which originates from a point mutation in the DELLA domain of the *GIBBERELLIC ACID INSENSITIVE* (*GAI*) gene (Boss and Thomas, 2002). This periclinal mutation, which is restricted to the L1 layer, makes the epidermis insensitive to GA, a hormone that regulates trichome formation in *Arabidopsis* (Gan et al., 2006; Perazza et al., 1998). Pinot Meunier had slightly higher nighttime transpiration than Pinot noir, with or without ABA (Fig. 3 B).

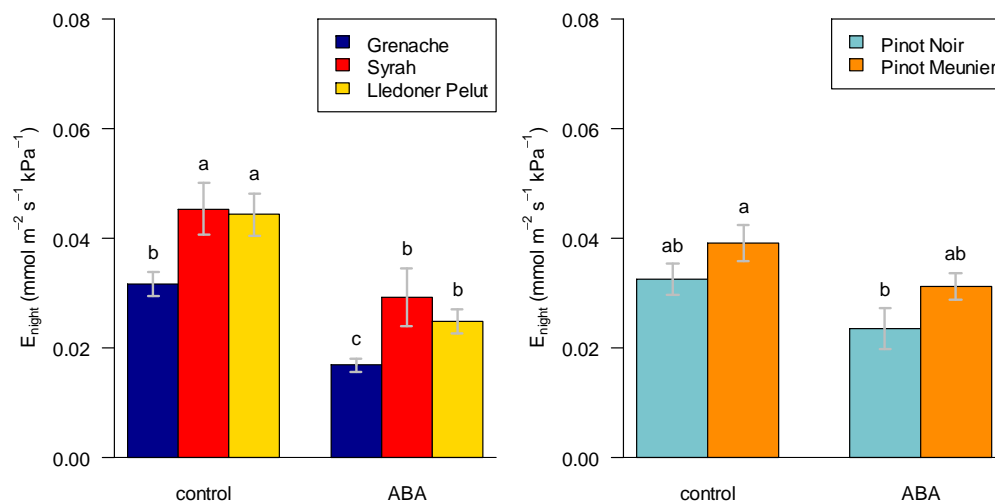


Figure 3: The effect of ABA on nighttime transpiration. Detached grapevine leaves of cultivars with different levels of hairiness were treated with and without 500 μ M ABA. A) comparison of nighttime transpiration of glabrous Grenache with hairy Syrah and Lledoner Pelut (somatic mutant of Grenache). B) comparison of nighttime transpiration of low-hairy Pinot Noir and high hairy density Pinot Meunier (somatic mutant of Pinot Noir).

To assess whether leaf hairs directly enhance water loss, we next manipulated the level of prostrate trichomes through a manual, mechanical depilation on the same subset of genotypes. Surprisingly, removing trichomes did not result in a reduction of water loss. When performed one day before transpiration analysis, the mechanical hair removal enhanced daytime as well as nighttime

transpiration in Syrah, irrespective of the presence of exogenous ABA (Fig. 4B, C, E, F). To a lesser extent, higher transpiration was also observed after a mock treatment on the glabrous Grenache (Supplementary). These results suggest that the mechanical treatment damaged the epidermis and generated novel, non-stomatal routes for water loss. Inspection of the leaf surface under a macroscope confirmed epidermal damages, especially on Syrah where pores with similar dimension as stomata were observed at the base of the removed hairs (Fig. 4C). The upsurge of transpiration by the mechanical treatment attenuated when depilation was performed three days before measurement (Fig. 4E, F); when performed two weeks before, depilation had virtually no effect on transpiration (Fig. 4E, F), which suggests the pores that had been opened by hair removal got clogged as observed under the macroscope (Fig. 4D), allowing water loss to recover the initial daytime and nighttime rates. Similar observations were obtained on Lledoner Pelut, Pinot Noir, Pinot Meunier, and an additional, independent variety, the strongly pubescent Macabeu (Supplementary). Collectively, these results confirmed that hairy leaves show higher nighttime transpiration than glabrous leaves, but failed in demonstrating that prostrate trichomes enhance water loss directly. Since the effect of exogenous ABA suggested a non-stomatal origin, we hypothesized that contrasting properties of the epidermal cuticle underlie the difference in nighttime transpiration between glabrous and pubescent leaves.

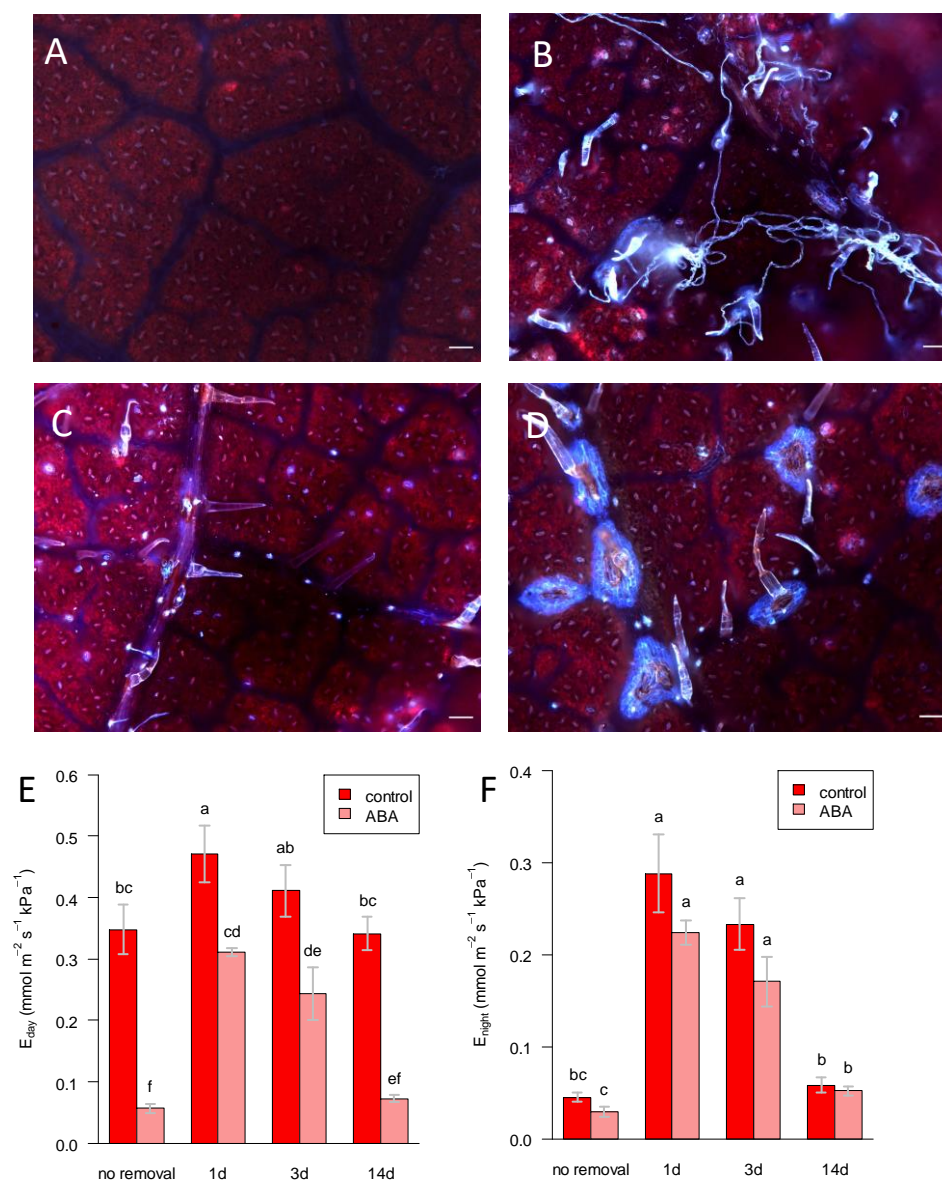


Figure 4: The effect of hair removal on day and nighttime transpiration in detached grapevine leaves. Pictures under the macroscope (bar represents 100 μm) show: glabrous Grenache (A), hairy Syrah with prostrate and erect trichomes (B), Syrah one day after hair epilation shows small spots on the nerves where trichomes were attached (C) and a Syrah leaf 14 days after epilation showing a patch around the spots where trichomes were mechanically removed. Daytime transpiration in detached leaves peeled either 1, 3 or 14 days before the experiment of cultivar Syrah with control and ABA solution (500 μM) (E). Nighttime transpiration for Syrah detached leaves peeled either 1, 3 or 14 days before the experiment. Errors bars represent standard error and statistical tests are done by Kruskal-Wallis.

Nighttime transpiration is genetically linked with properties of the cuticle, but not with stomatal density. To address the possibility that cuticular properties are linked to nighttime water loss, we explored the micromorphology of the epidermis, taking advantage of the existing variation for leaf cuticular nanoridges, also termed striae. Nanoridges are thought to arise from a relative excess of cuticle secretion compared to cell expansion, generating compressive forces that drive polar deposition of cuticle folds depending on the principal direction of surface growth (Hong et al., 2017; Kourounioti et al., 2013). They are most often found on the epidermis of floral organs, where they are involved in light diffraction for pollinator attraction (Kay et al., 1981; Whitney et al., 2009). They may also be found in other organs of some species, like in the leaves of the *Vitis* genus (Ickert-Bond et al., 2018; Konlechner and Sauer, 2016), where their role remains elusive. To analyse the variation in cuticular nanoridges in our association panel, we obtained epidermal imprints using nail polish applied on a depilated region of the abaxial side of leaves. Occasionally the nail polish layer was immediately absorbed and could not be retrieved, so that it was necessary to apply dental paste before gathering the imprint. We observed a large variability for cuticular striae in our population (Fig. 5): some varieties were devoid of striae, with a perfectly smooth epidermis; others showed low to moderate presence of striae, essentially radiating from stomata and trichomes; and others showed a strong density of striae covering the whole epidermis. We performed a GWAS on the striae score, and recovered the same SNP on chromosome 5 as for nighttime transpiration and trichomes. At this locus, larger abundance of striae was associated with higher nighttime transpiration and more trichomes. We then ran another GWAS based on the requirement of dental paste for obtaining imprints, as a proxy for cuticle permeability to nail polish (Suppl. Fig. 6). Again, we found the same SNP on chromosome 5, with more permeable cuticles having more abundant striae, suggesting the latter were associated to a change in cuticle composition and function.

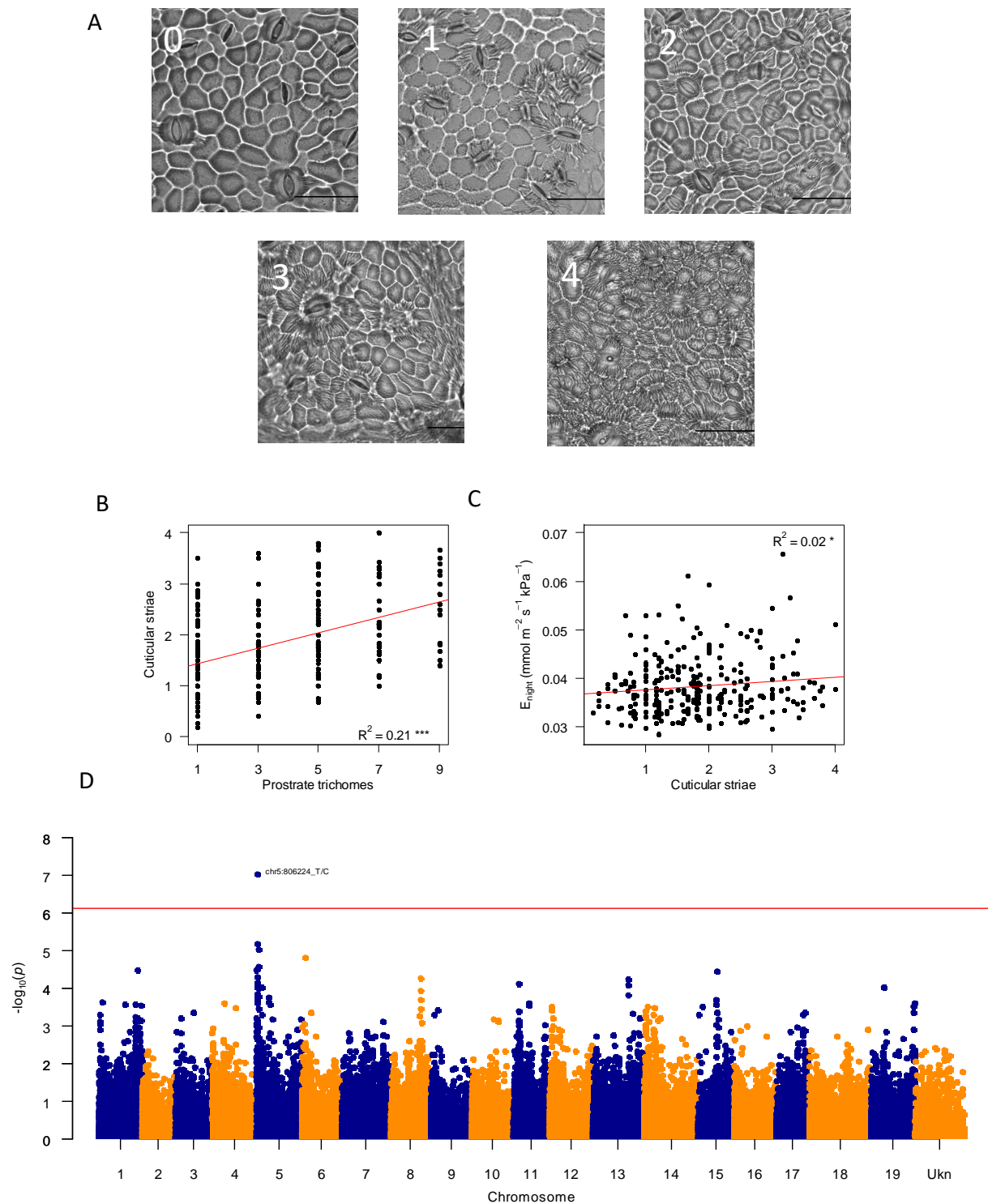


Figure 5: Presence of nanoridges on the abaxial side of grapevine leaves. A) a score was given for the intensity of the presence of nanoridges from low to zero (0) up to 4 (high). B) correlation between the level of prostrate trichomes and nanoridges including the coefficient of variation R^2 . C) correlation between nanoridges and nighttime transpiration. D) GWAS for the density of nanoridges on the leaf.

That the presence of cuticular striae is associated to an increase in nighttime transpiration at the SNP is somewhat puzzling. Arabidopsis mutants devoid of nanoridges in epidermal cells of floral organs show increased, not decreased, permeability therein (Bessire et al., 2011; Fabre et al., 2016; Hong et

al., 2017; Li-Beisson et al., 2009; Oshima et al., 2013; Panikashvili et al., 2011). However, nanoridges are made of cutin, while cuticular waxes are the actual barrier for water transport (Riederer and Schreiber, 2001). Cutin may affect epidermal permeability only in an indirect way, since its structural organization modulates the waterproof capacity of the intracuticular waxes that are embedded in the cutin polymer (Li et al., 2007). Accordingly, there is no consistent relationship between cutin content and epidermal water permeability. In tomato, three non-allelic mutants severely deficient in cutin content (less than 5% of the wild-type) had either similar or twofold rate of water loss compared to the wild-type (Isaacson et al., 2009). Moreover, both deficiency and excess of cutin production may result in a decreased epidermal permeability, as shown by the manipulation of genes of the cutin metabolism in *Arabidopsis* (Li et al., 2007). Similarly, overexpressing of *SHINE* AP2/ERF transcription factors in *Arabidopsis* increases the production of cutin and wax (Kannangara et al., 2007) but enhances the leaf epidermal permeability and the rate of water loss in detached rosettes (Aharoni et al., 2004), while knocking down *SHINE* expression reduces cutin and wax content, and consistently enhances epidermal permeability (Oshima et al., 2013; Shi et al., 2011). Similar conclusions could be drawn from *SHINE* manipulation in tomato leaves and fruits (Buxdorf et al., 2014; Shi et al., 2013). Thus, cutin nanoridges in grapevine leaves may witness a change in the composition or organization of wax, which would enhance epidermal permeability and nighttime water loss.

The possibility that cuticular nanoridges were related to a change in stomatal properties was also examined, yet. For instance, an impairment in cutin or wax metabolism may affect stomatal transpiration if it affects the formation of the cuticular ledge in the guard cells (Li et al., 2007; Tang et al., 2020). Alternatively, cuticular nanoridges around stomata could mechanically prevent the guard cells to fully close upon darkness. However, monitoring stomatal dynamics in response to step changes in irradiance did not reveal differences between Grenache, Lledoner pelut and Syrah (Suppl. Fig. 11), ruling out both propositions. Nonetheless, the observation that cuticular nanoridges essentially radiate from stomata – and trichomes – in grapevine leaves strongly suggest that these structures share part of their developmental programs. This idea was supported by the observation that *SHINE* overexpression leads to a severe reduction of stomatal density (Aharoni et al., 2004), while *SHINE* silencing modifies the expression of several genes involved in epidermal patterning (Shi et al., 2013). To explore the hypothesis that differences in stomatal development supports contrasting rates of transpiration at night at the SNP detected on chromosome 5, we performed a GWAS on stomatal density. We could not identify any significant association for this trait (Suppl. Fig. 5), suggesting stomatal development is not involved in the control of nighttime transpiration at the SNP on chromosome 5. It should be noted, however, that the visual identification of stomata in our imprints was particularly difficult on the imprints collected from the pubescent cultivars, yet they had been depilated.

The R2R3-MYB transcription factor MYBF2 most likely controls epidermal morphogenesis. Three candidate genes related to epidermal development were found in the 30 kb surrounding the SNP: *ZFP6* (VIT_05s0077g01390), *MYBF2* (VIT_05s0077g01360) and *TBL4* (VIT_05s0077g01340). Investigating the expression patterns in available databases (Fasoli et al., 2012; Velt and Rustenholz, 2020) revealed that *ZFP6* was mostly expressed in the tendril (Suppl Fig. 7), a stem-like organ that develops from the same meristem as the inflorescence and that is promoted by GA (Carmona et al., 2008). This is reminiscent of the *Arabidopsis* *ZFP6*, that integrates GA signalling to act mainly in inflorescence stem (Zhou et al., 2013). Thus, *ZFP6* does not appear as a strong candidate. By contrast, *MYBF2* and *TBL4* showed the strongest expression in bud outgrowth and young leaves, respectively (Suppl Fig. 8,9), which correspond to the expected stages for the development of epidermal structures. *MYBF2* was also expressed in the berry pericarp, including skin (Suppl. Fig. 9). We then examined the list of co-expressed genes for the three candidates using the ViTis Co-expression database (Wong et al., 2013)

expanded for more than 1000 samples (Darren Wong, pers. comm.). Note that the dataset was still expanded to 1359 samples (Wong, 2020) but released only one week before submitting the manuscript, thus we only refer to the 1000-sample dataset unless stated otherwise. *ZFP6* and *TBL4* were co-expressed with only a few genes putatively involved in trichome formation or cuticle (Suppl. Table 2,3). By contrast, *MYBF2* was co-expressed with many genes of high relevance for epidermal morphogenesis (Suppl. Table 4), making it the most likely candidate as described thereafter.

MYBF2 belongs to a small subclade of R2R3-MYB transcription factors typically involved in the transcriptional regulation of flavonol metabolism (Matus et al., 2008; Wong et al., 2016). While its sole grapevine homolog *MYBF1* has an established role in regulating berry flavonol metabolism, *MYBF2* lacks conserved motifs shared by other *MYB* flavonol regulators in several species, and the contribution of *MYBF2* to flavonol metabolism remains elusive (Czemmel et al., 2009; Matus et al., 2009). *MYBF2* co-expression analysis revealed several genes relevant to flavonoid metabolism (Suppl. Table 3), and we could recover *FLAVONOL SYNTHASE 1* (VIT_18s0001g03510; detected using the 1359 samples dataset), which is activated by *MYBF1* to trigger flavonol biosynthesis during berry development (Czemmel et al., 2009). Moreover, many other genes captured our attention that were related to epidermal morphogenesis. We recovered a series of transcription factors with recognized roles on trichome and/or cuticle development: *MIXTA3/MYB142* (VIT_17s0000g06410, rank #17) and *MIXTA1/MYB140* (VIT_01s0026g02770, rank #61), two members of the MIXTA-like R2R3-MYB family, which is involved in cuticle and trichome formation in many species, including Arabidopsis where the *MIXTA* genes *AtMYB106/NOECK* and *AtMYB16* control cuticular nanoridges of petal cells partly by activating *SHINE1* (Galdon-Armero et al., 2020; Glover et al., 1998; Lashbrooke et al., 2015; Noda et al., 1994; Oshima and Mitsuda, 2013; Oshima et al., 2013; Perez-Rodriguez, 2005; Ying et al., 2020); *MYBT1/MYB155* (VIT_01s0127g00730, rank #44), a member of the small *MYB* subclade that contains *AtMYB0/GLABRA1*, *AtMYB23* and *AtMYB66/WEREWOLF* (Wong et al., 2016), which regulate epidermal cell fate into trichomes or root hairs (Ishida et al., 2008); and *JAGGED* (VIT_01s0011g03600, rank #48), a zinc finger transcription factor whose loss-of-function mutant shows several developmental defects, including irregular cuticular nanoridges in the petal cells (Dinneny et al., 2004), which may arise from a loss of organ polarity therein (Sauret-Güeto et al., 2013). Furthermore, several co-expressed genes were involved in cuticle production and nanoridge formation: a member of the wax ester synthase/diacylglycerol acyltransferase family (VIT_16s0098g00380, rank #1), which is involved in the metabolism of cuticular waxes (Dimopoulos et al., 2020; Li et al., 2008; Patwari et al., 2019) and nanoridges in Arabidopsis petals (Takeda et al., 2013); two ABC transporters VIT_06s0061g00250 (rank #9) and VIT_06s0061g00260 (rank #145), orthologous to Arabidopsis *ABCG13/FOLDED PETALS 2* and *ABCG12/ECERIFERUM5*, respectively, which contributes to the extracellular secretion of cuticular lipids (Panikashvili et al., 2011; Pighin et al., 2004), and to the formation of nanoridges in petals for *ABCG13* (Panikashvili et al., 2011; Takeda et al., 2014); several members of the β -ketoacyl-CoA synthase (VIT_16s0022g01580, rank #16; VIT_16s0022g02190, rank #36; VIT_00s0179g00380, rank #40; VIT_01s0011g03490, rank #141; VIT_16s0022g01570, rank #210) and reductase (VIT_01s0137g00130, rank #227; VIT_01s0137g00160, rank #255) families, involved in the biosynthesis of fatty acids and wax (Dimopoulos et al., 2020); three orthologs of *ECERIFERUM1* (VIT_15s0045g01460, rank #43; VIT_15s0045g01440, rank #236; VIT_15s0045g01520, rank #253), which is involved in the biosynthesis of alkanes for cuticular waxes (Bourdenx et al., 2011); two members of the cytochrome P450 families 96 (VIT_07s0031g01670, rank #47) and 77 (VIT_10s0116g00800, rank #65), which are close homologs of Arabidopsis *CYP96A15/MID-CHAIN ALKANE HYDROXYLASE 1* (Greer et al., 2007) and *CYP77A6* (Li-Beisson et al., 2009), involved in cuticular wax and cutin polyester biosynthesis, respectively, and nanoridges formation in petals for *CYP77A6* (Li-Beisson et al., 2009); and several members of the largely-uncharacterized superfamily of GDSL-lipases, which contains genes involved in the formation

of the cuticle (Yeats and Rose, 2013), its nanoridges (Hong et al., 2017), and the outer cuticular ledge of the stomatal pore (Tang et al., 2020). Interestingly, we also found numerous co-expressed genes related to stomatal development, including a homolog of *MUSTACHES* (*MUS*, VIT_18s0001g13590, rank #6), a leucine-rich repeat receptor-like kinase that regulates stomatal symmetry (Keerthisinghe et al., 2015); *SPEECHLESS* (*SPCH*, VIT_16s0050g02500, rank #11), the basic helix-loop-helix transcription factor that initiates stomatal lineage (MacAlister et al., 2007); three homologs of *EPIDERMAL PATTERNING FACTOR 1* (*EPF1*, VIT_19s0014g03220, rank #15), *EPF2* (VIT_18s0164g00010, rank #24) and *EPF LIKE-9/STOMAGEN* (*EPFL9*, VIT_05s0062g01380, rank #71), which encode secretory peptides responsible of proper stomatal spacing through intercellular communication and to a lesser extent *TOO MANY MOUTHS* (*TMM*, VIT_09s0002g07030, rank #197), a leucine-rich repeat containing receptor-like protein also involved in stomatal patterning. Thus, it could be that *MYBF2* is preferentially induced in the guard cells and the trichomes, generating covariation in the expression pattern. Collectively, these data strongly suggest that *MYBF2* connects several aspects of epidermal morphogenesis with flavonoid metabolism. Preliminary sequence analyses on publicly available genomic data (genome browser JBrowse) revealed a deletion within exon 3 of *MYBF2* that was shared between glabrous genotypes. Further genomic analyses on contrasting grapevine genotypes will be necessary to ascertain this hypothesis.

Discussion

Reducing transpiration at night is an appealing strategy to breed plants for more efficient use of water (Coupel-Ledru et al., 2016). At the leaf epidermis, stomata (Hetherington and Woodward, 2003), cuticle (Kerstiens, 1996; Riederer and Schreiber, 2001) and to a lesser extent trichomes (Bickford, 2016; Johnson, 1975) have all been involved in the regulation of water loss. During the day, leaves essentially transpire through open stomata. At night, stomata close partly, thereby enhancing the relative contribution of the cuticle, and potentially trichomes, to water loss (Caird et al., 2007). The morphogenesis of these epidermal structures is tightly intertwined. Many mutants of the cuticle biosynthetic pathway show defects in trichome and/or stomatal development (Bird and Gray, 2003). Reciprocally, several trichome mutants are affected in properties of the cuticle (Xia et al., 2010). Moreover, to some extent, trichomes and stomata compete for epidermal space, and negative correlations may be observed between trichome and stomatal densities (Galdon-Armero et al., 2018). Yet, the mechanistic bases for these relationships are not fully understood. Shared morphogenetic events typically involve transcription factors that directly control the expression of several target genes. Accordingly, several MIXTAs have been shown to regulate the development of both cuticle and trichomes in several species (Galdon-Armero et al., 2020; Glover et al., 1998; Lashbrooke et al., 2015; Oshima and Mitsuda, 2013; Oshima et al., 2013; Perez-Rodriguez, 2005), as well as fibre development in cotton (Machado et al., 2009; Walford et al., 2011). Other TCP transcription factors have recently been proposed to act upstream of the Arabidopsis MIXTA AtMYB106 in controlling cuticle formation and trichome differentiation (Camoirano et al., 2020). Here, we identified a major association for nighttime transpiration that connects the different facets of epidermal morphogenesis in grapevine leaves, most likely through the *MYBF2* transcription factor.

Using GWAS, we uncovered an SNP that was significantly associated with nighttime transpiration, prostrate trichomes and cuticular nanoridges, and co-expression analyses strongly suggested that *MYBF2* is the causal gene for all these phenotypes. At this locus, the allele showing higher water loss also conferred higher abundance of trichomes and nanoridges. While the presence of trichomes might increase the surface exchange available for water evaporation to the atmosphere and thus increase water loss, depilation experiments did not support this hypothesis in our conditions. While the

presence of cuticular nanoridges in *Arabidopsis* floral organs has always been associated with reduced cuticle permeability (Bessire et al., 2011; Fabre et al., 2016; Hong et al., 2017; Li-Beisson et al., 2009; Oshima et al., 2013; Panikashvili et al., 2011), we propose that cuticular ridges of grapevine leaves mirror a deep change in cuticle composition or organisation that increases its permeability despite enhancing its local abundance. Candidate genes for such a scenario could be the *SHINE* transcription factors, which increase the production of cutin and wax yet enhance leaf water loss when overexpressed (Aharoni et al., 2004; Kannangara et al., 2007). Interestingly, *SHINE1* is upregulated by AtMYB106 (Oshima et al., 2013), encoded by a close *Arabidopsis* homolog of grapevine *MIXTA3* and *MIXTA1*, which are co-expressed with *MYBF2* in grapevine. It should be noted, however, that none of the three *SHINE* homologs could be recovered in the first 300 genes co-expressed with *MYBF2*.

An alternative yet non-exclusive hypothesis is that *MYBF2* controls cuticular permeability by enhancing flavonoid biosynthesis. The subclade that hosts *MYBF2* and its homolog *MYBF1* contains genes typically involved in flavonol metabolism (Wong et al., 2016), including the *PRODUCTION OF FLAVONOL GLYCOSIDES (PFG)* genes *AtMYB12/PFG1*, *AtMYB11/PFG2*, and *AtMYB111/PFG3* initially identified in *Arabidopsis* (Mehrtens et al., 2005; Stracke et al., 2007). *MYBF1* activates flavonol metabolism (Czemmel et al., 2009), and our co-expression analysis makes plausible the involvement of *MYBF2* in flavonoid metabolism. In tomato, mutations of *SlMYB12* affect the flavonoid pathway in the fruit peel, which generate pink fruit color (Adato et al., 2009; Ballester et al., 2010; Fernandez-Moreno et al., 2016; Zhu et al., 2018), and expression of *Arabidopsis AtMYB12* in tomato fruits also activates larger metabolic pathways (Luo et al., 2008; Zhang et al., 2015). Interestingly, flavonoids normally accumulate in the cuticle of tomato fruits during ripening, which correlates with a mechanical reinforcement of the cuticle that is not observed in flavonol-deficient mutants (Domínguez et al., 2009). Downregulating flavonoid metabolism through silencing of *CHALCONE SYNTHASE (CHS)* in tomato fruits resulted in a looser cuticle but less permeable through a negative effect on wax accumulation (España et al., 2014; Heredia et al., 2015). Nonetheless, a pink tomato mutant impaired in *SlMYB12*, showing down-regulation of *CHS* expression and deficiency of flavonoid accumulation, did not reveal decreased cuticle permeability or reduced fruit water loss (Adato et al., 2009). Thus, it remains to be established whether *MYBF2* enhances cuticular water loss by promoting flavonoid biosynthesis and/or by triggering profound changes in the molecular arrangement of the cuticle.

Beyond the cuticle, the co-expression profile of *MYBF2* pointed to a strong link with trichomes and stomata – yet none of them were likely involved in the control of nighttime transpiration through *MYBF2*, as suggested by the depilation experiment and the GWAS on stomatal density. The observation that cuticle nanoridges essentially radiates from stomata and trichomes intimates shared morphogenetic events between these structures. One scenario could be that *MYBF2* is induced in the precursor cells of stomata and trichomes as patterned by homologs of *SPCH* (MacAlister et al., 2007) and *GL1* (Ishida et al., 2008), respectively, which were recovered in the co-expressed genes (Figure 6). The activation of *MYBF2* would then upregulate the cutin biosynthesis pathway around these cells, possibly with a range that depends on the mobility of the EPF peptides (Zeng et al., 2020) encoded by the *EPF* genes co-expressed with *MYBF2*. Then the areal growth of pavement cells, essentially isotropic when epidermal cells start differentiating (Fox et al., 2018), would generate a radiating pattern of cuticular nanoridges around stomata and trichomes, marking the cells with stiffer cuticle. On the other hand, misregulation of wax deposition would make the epidermis of those cells more permeable. *MYBF2* is also likely to interact with *MIXTA* in specifying the prostrate trichome identity. *MYBF2* could be inhibited by GA through the degradation of DELLA proteins like its *Arabidopsis* homologs (Tan et al., 2019). This would actually explain why GA inhibits trichome production in grapevine (Boss and Thomas, 2002), but not in *Arabidopsis* where its homologs are not involved trichome initiation (Tan et al., 2019). Overall, these interpretations fit with a framework where *MYBF2* connects the different facets of leaf

epidermal morphogenesis, promoting the formation of prostrate trichomes, cutin nanoridges around trichomes and stomata, and permeability of the cuticle that enhances nighttime transpiration.

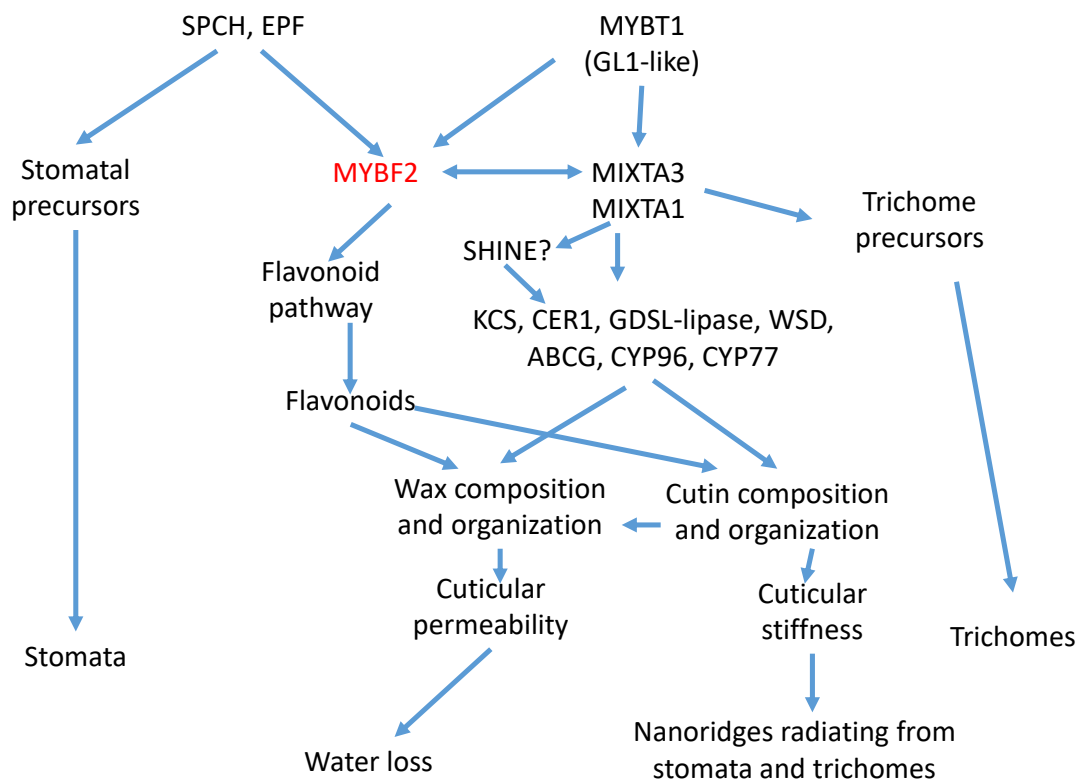


Figure 6: Preliminary framework of the possible interactions of MYBF2 during epidermal development. MYBF2 is probably activated downstream of SPCH, not directly interfering with stomatal development. MYBF2 is expected to interact with MIXTA3 may lead to the production of prostrate trichomes and cuticular nanoridges. The permeability of the cuticle is not expected to be altered by nanoridges itself but via the biosynthesis and secretion of epicuticular waxes.

We propose that neofunctionalization of *MYBF2* has been selected during grapevine domestication. While the other MYBs of its subclade are specialized into flavonoid metabolism, our data suggest *MYBF2* has acquired novel functions related to epidermal morphogenesis. It is tempting to speculate that the production of trichomes – the only trait visible by eye – has been first selected as a fence against pathogens (Kortekamp and Zyprian, 1999). In line with this assertion, a QTL for prostrate trichomes, corresponding to our SNP on chromosome 5, co-localized with a QTL of downy mildew resistance (Divilov et al., 2017; Kono et al., 2018). Nonetheless, cuticle properties also affect other aspects of plant defense in several ways, e.g. through pathogen sensing (Bessire et al., 2007) or transmission of the systemic acquired resistance (Lim et al., 2020; Xia et al., 2009, 2010). Thus, cuticular nanoridge may also somehow contribute to improve grapevine defense. In the context of climate change, our study paves the way for designing strategies to uncouple the different facets of epidermal morphogenesis so as to reduce the leaky effect of *MYBF2* on nighttime transpiration while retaining its defense properties.

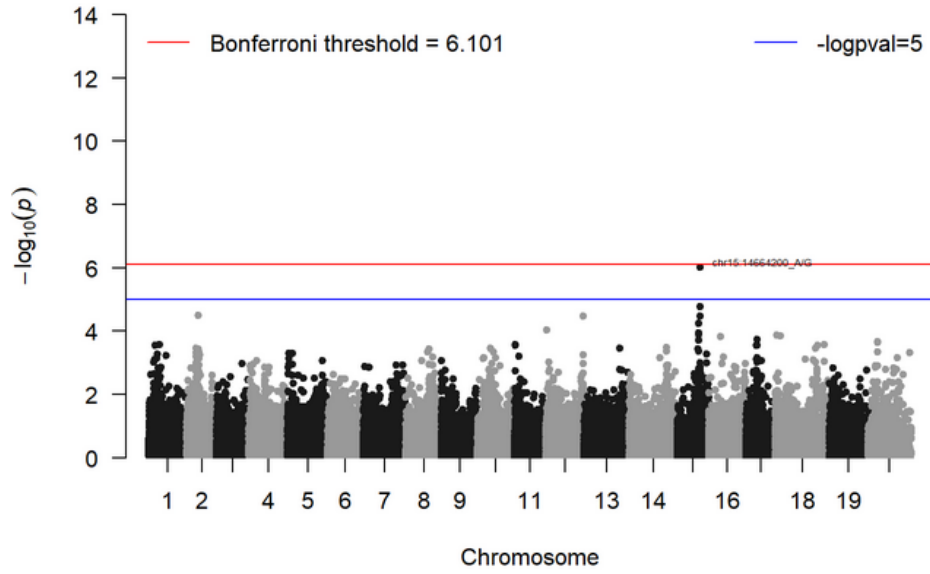
Acknowledgements

We thank the teams AGAP, DAAV and Vassal for help in preparing the plants of the association panel. Thanks to JM Boursiquot and T Lacombe for advices on selecting varieties and scoring trichomes. Thanks to Philippe Péchier and Freddy Gavanon for taking care of the plants in the nursery. Thanks to the Pierre Galet Vineyard of SupAgro and people from SupAgro handling the plants. Myriam for handling PHENOPSIS. Carine Alcon for microscopy. Jasper Lamers for help with leaf area analysis. Stephane Berthézene for installation and registration of balances.

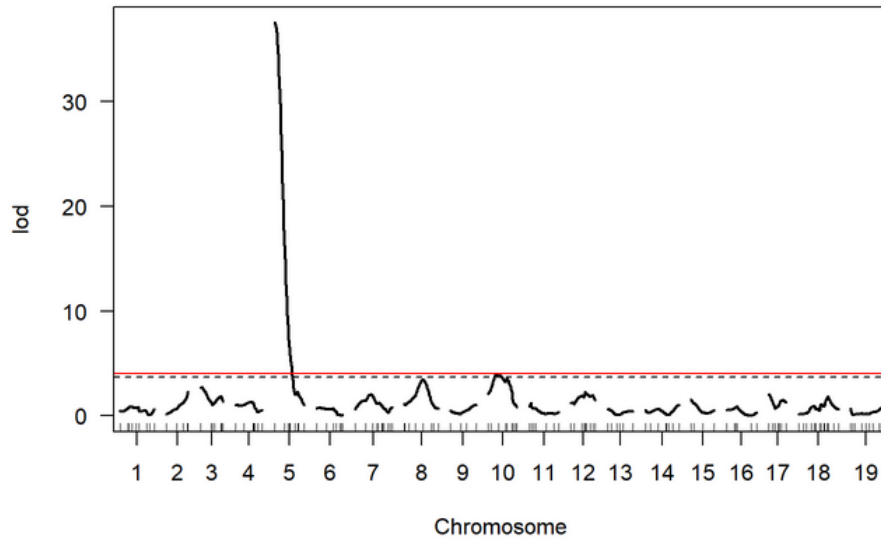
Supplementary

Table 1: candidate genes surrounding the SNP

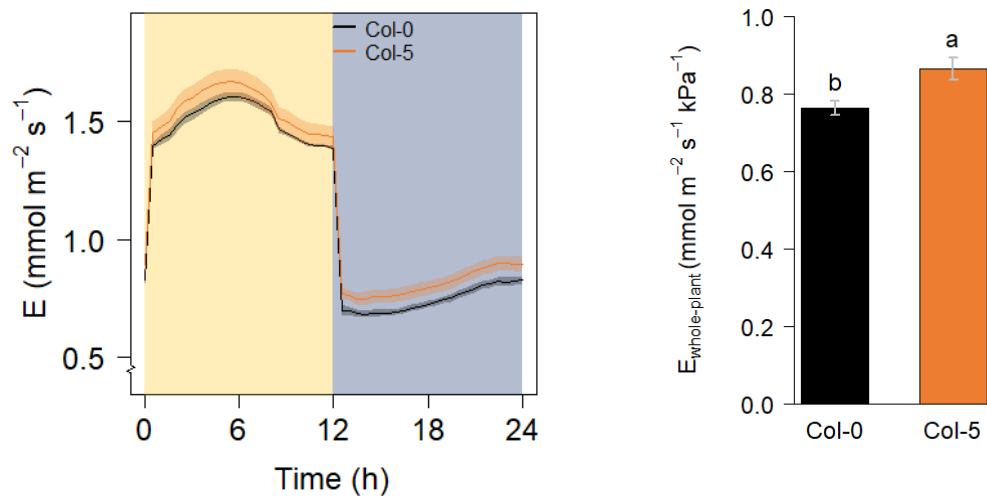
vitvi.geneid	refseq.genedesc	gene.start_IGGP12Xv2	gene.end_IGGP12Xv2
Vitvi05g01752	pathogenesis-related protein 10.5	615641	616290
Vitvi05g00069	NA	623120	623736
Vitvi05g01753	major allergen Dau c 1-like	628112	630001
Vitvi05g01755	NA	642853	643583
Vitvi05g00070	NA	645697	645943
Vitvi05g01756	pathogenesis-related protein 10	647719	648563
Vitvi05g00071	major allergen Pru ar 1-like	651365	652300
Vitvi05g01758	pathogenesis-related protein 10.3-like	664670	665619
Vitvi05g01759	pathogenesis-related protein 10.3-like	669035	669956
Vitvi05g00072	major allergen Pru av 1-like	672346	672947
Vitvi05g01760	major allergen Pru av 1	679517	680357
Vitvi05g01761	pathogenesis-related protein STH-2	683354	683938
Vitvi05g00073	uncharacterized LOC100258588	688685	707778
Vitvi05g01762	protein DEHYDRATION-INDUCED 19 homolog 4	709124	714054
Vitvi07g02622	transfer RNA threonine (anticodon AGU)	714686	714759
Vitvi05g00074	uncharacterized LOC104879211	715323	717756
Vitvi05g00075	two-component response regulator ARR1	723923	728378
Vitvi05g00076	COBRA-like protein 7	729622	733533
Vitvi05g01763	leucine-rich repeat extensin-like protein 3	739921	740917
Vitvi05g00077	GATA transcription factor 16	747538	748871
Vitvi05g00078	NA	755834	756432
Vitvi05g01764	NA	756718	757119
Vitvi05g01765	importin subunit alpha-2	759023	763209
Vitvi05g00079	uncharacterized LOC100260115	766503	768097
Vitvi05g00080	NA	769249	769503
Vitvi05g00081	protein FAR1-RELATED SEQUENCE 11	770809	777314
Vitvi05g00082	zinc finger protein 6-like	782616	783734
Vitvi05g01766	NA	787542	787868
Vitvi05g00083	dentin sialophosphoprotein	790152	796515
Vitvi05g00084	transcription factor MYB12	805550	807839
Vitvi05g00085	AMSH-like ubiquitin thioesterase 3	813097	824247
Vitvi05g00086	protein trichome birefringence-like 4	824860	826658
Vitvi05g01767	uncharacterized LOC100241383	827476	828495
Vitvi05g00087	putative pentatricopeptide repeat-containing protein At3g16890, mitochondrial	834778	836919
Vitvi05g00088	NA	839007	839156
Vitvi05g01768	3-oxo-Delta(4,5)-steroid 5-beta-reductase-like	839283	840699
Vitvi05g01769	3-oxo-Delta(4,5)-steroid 5-beta-reductase-like	843543	844995
Vitvi05g01770	uncharacterized LOC100255126	849476	854019
Vitvi05g00089	putative beta-D-xylosidase	858035	863113
Vitvi05g00090	acetate/butyrate--CoA ligase AAE7, peroxisomal	868859	873471
Vitvi05g00091	NA	876471	876732
Vitvi05g00092	acetate/butyrate--CoA ligase AAE7, peroxisomal	886487	891593
Vitvi05g00094	chitinase-like protein 2	893908	896239
Vitvi05g00095	NA	904489	906501
Vitvi05g00096	calmodulin-binding transcription activator 6	912778	925810
Vitvi05g00097	GTP-binding protein SAR1A	927041	929500
Vitvi05g00098	60S ribosomal protein L29	931580	932183
Vitvi05g00099	NA	933515	933776
Vitvi05g00100	dihydrolipoyl dehydrogenase 2, chloroplastic	939049	947811
Vitvi05g00101	serine/threonine-protein phosphatase 4 regulatory subunit 3A	954968	971936
Vitvi05g00102	outer envelope pore protein 16-2, chloroplastic	972438	975129
Vitvi05g00104	zinc finger CCHC domain-containing protein 10	976037	978593
Vitvi05g00105	DNA-directed RNA polymerases II, IV and V subunit 9A	979563	984223
Vitvi05g00106	bromodomain and WD repeat-containing protein 3	984657	1007813



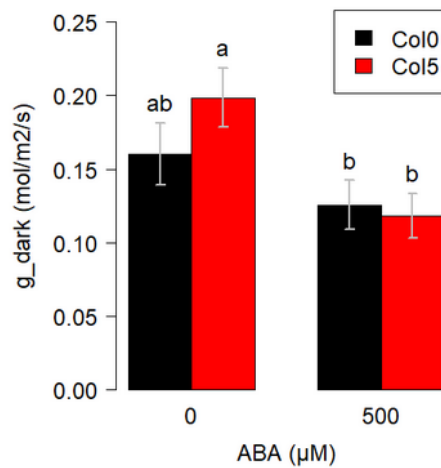
Supplementary figure 1: Manhattan plot on the density of erect trichomes located in the leaf lamina.



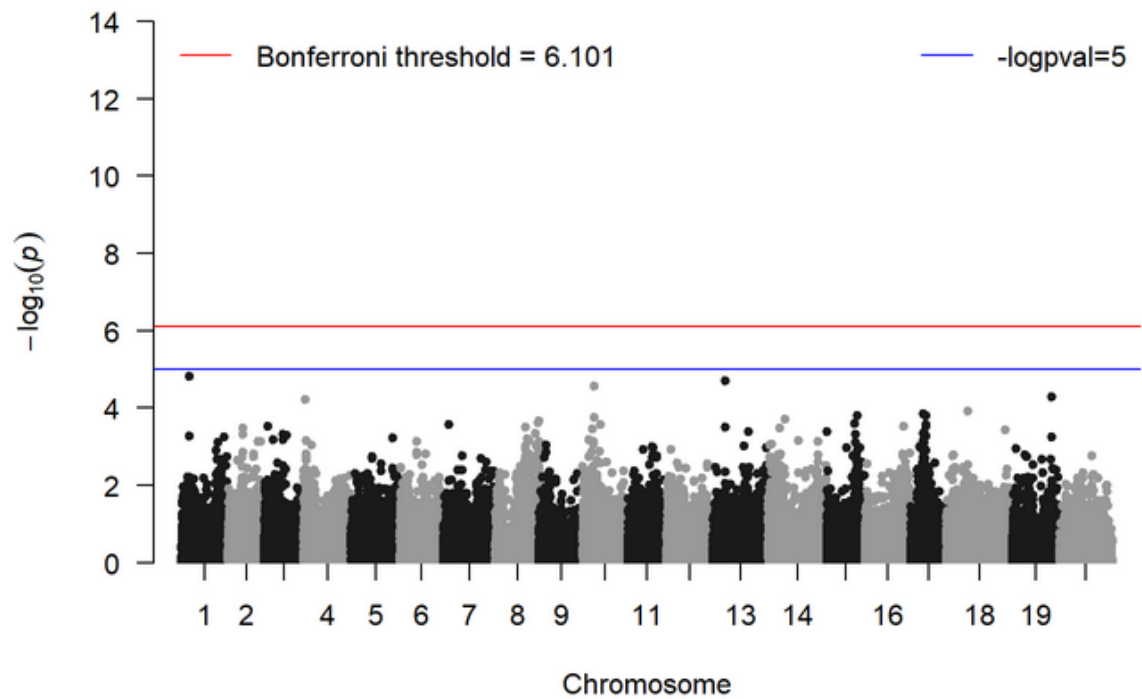
Supplementary figure 2: QTL analysis for prostrate trichome density in a mapping population of Syrah x Grenache.



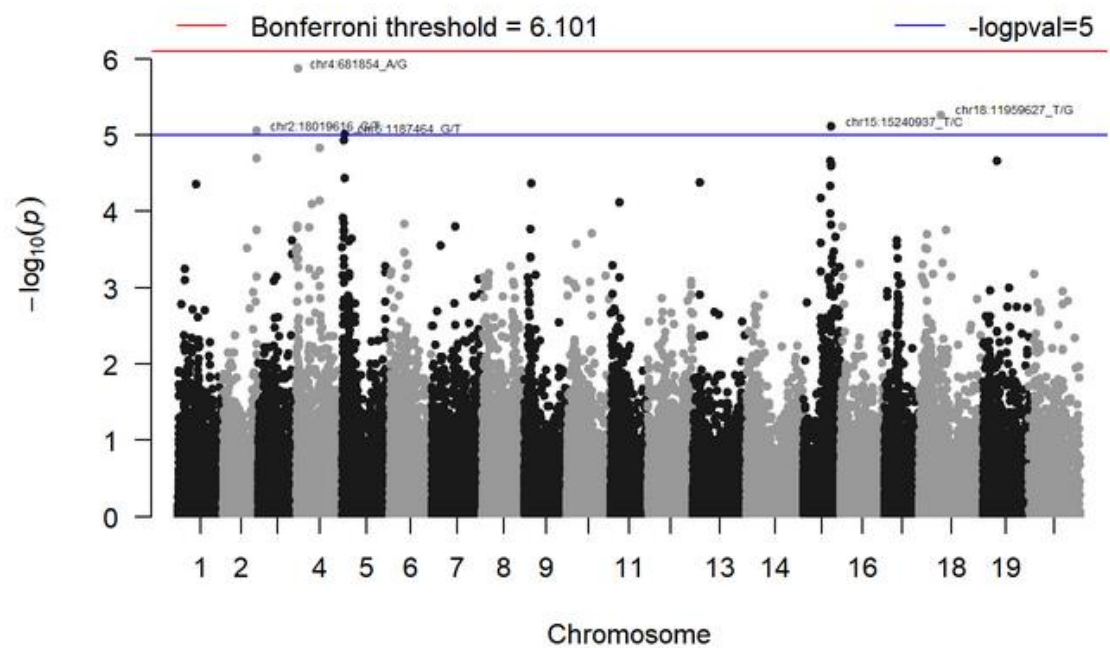
Supplementary figure 3: Diel transpiration patterns were calculated in whole-plants of *Arabidopsis thaliana* Col-0 (hairy) and Col-5 (glabrous). Every treatment contained five replicates.



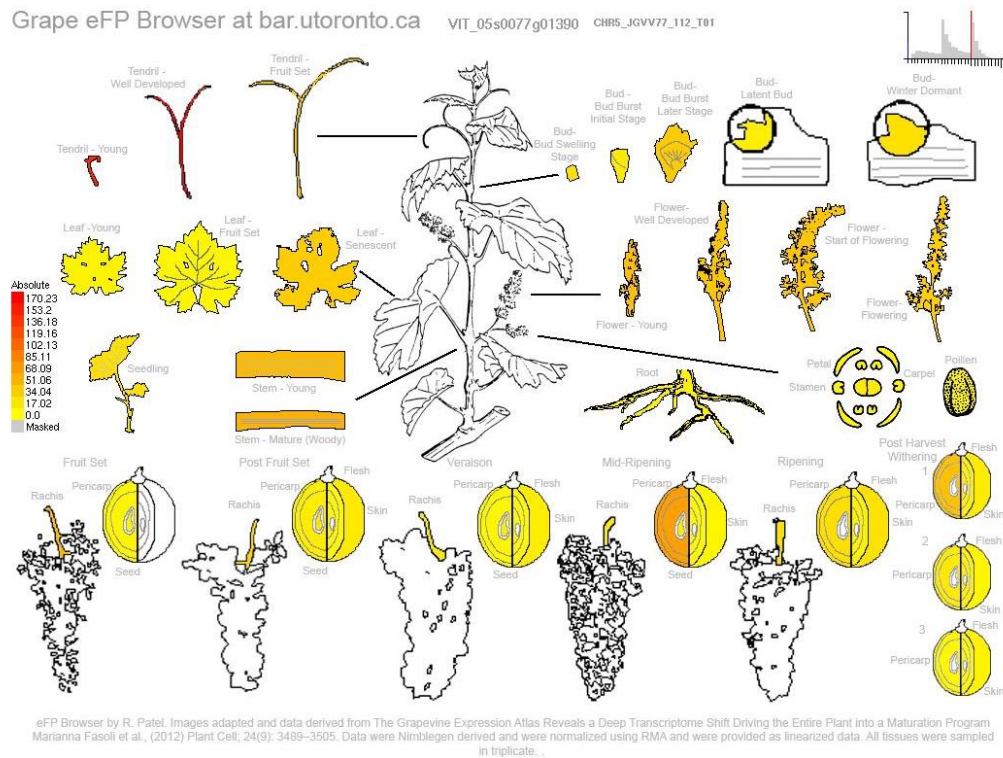
Supplementary figure 4: Nighttime stomatal conductance in detached leaves of *Arabidopsis thaliana* Col-0 (hairy) and Col-5 (glabrous). Leaves were infused with a high dose of abscisic acid (ABA) and measured after full stomatal closure. Every treatment had five replicates.



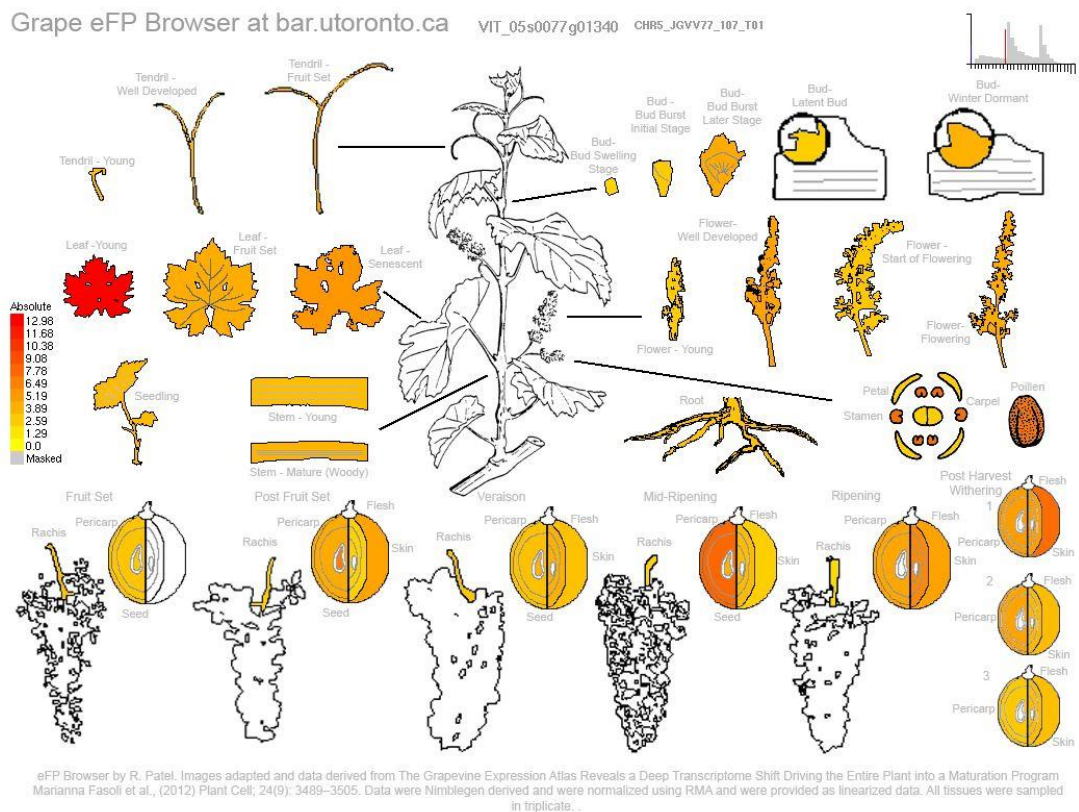
Supplementary figure 5: GWAS result on stomatal density in the grapevine association panel. Thresholds correspond to Bonferroni (red) and $-\log(5)$.



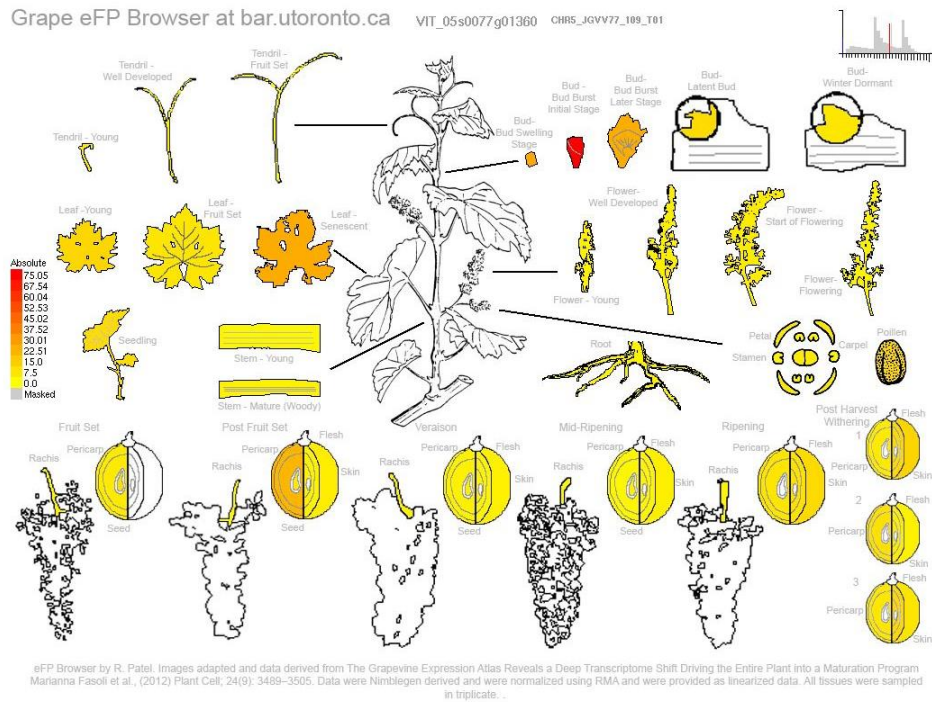
Supplementary figure 6: GWAS result on a proxy for epidermal permeability to nail polish during stomatal imprints in the grapevine association panel. Thresholds correspond to Bonferroni (red) and $-\log(5)$.



Supplementary figure 7: Expression results of the abundance of transcripts for ZFP6 from (Fasoli et al., 2012).



Supplementary figure 8: Expression results of the abundance of transcripts for TBL44 from (Fasoli et al., 2012).



Supplementary figure 9: Expression results of the abundance of transcripts for MYBF2 from (Fasoli et al., 2012).

Table 2: Co-expression results for ZFP6 from the VTCdb (Wong et al., 2013).

☒ Hide/show additional information on co-expressed genes
☐ Show all co-expressed genes

Target	Coex Query	Coex Query	Coex Query	Symbol	PCC	All	Berry	Stress	Functional annotation	Psort	Vitisnet molecular network
VIT_18s0001g12870	All	Berry	Stress	CYP722A1	0.78018	3	417	60	CYP722A1	C N CY CYS/N C/M CY/PM EX	ND
VIT_14s0060g01180	All	Berry	Stress	KNAT6_2	0.80106	4	294	383	KNAT2 (knotted1-like homeobox gene 6)	N	vv60034HB
VIT_18s0001g03160	All	Berry	Stress	MTN21	0.77091	9	497	1030	Nodulin MTN21 family	C PM C/M	vv50122Porters cat 7 to 17
VIT_08s0040g01240	All	Berry	Stress	BHLHDOM_54	0.75437	11	378	802	Basic helix-loop-helix (bHLH) family	N CY/N	vv600118HLH
VIT_02s0025g02710	All	Berry	Stress	NST1_2	0.77792	11	66	9	NAC Secondary wall thickening promoting factor1	N	vv60046NAC
VIT_14s0083g00150	All	Berry	Stress	TCP1_1	0.75286	13	641	9128	TCP family transcription factor 1	N	vv60061TCP
VIT_08s0007g05740	All	Berry	Stress	BOP2_1	0.75725	13	415	110	BLADE-ON-PETIOLE2	ER C N VM CYS/N M PM M/PM CY/PM CY	vv60093TRAF
VIT_07s0104g01820	All	Berry	Stress	GSTF7	0.75126	14	471	137	Glutathione S-transferase 11 GSTF7	CY	vv10480Glutathione metabolism
VIT_01s0011g05410	All	Berry	Stress	PMEI	0.76287	15	296	78	Invertase/pectin methylesterase inhibitor	EX C	vv40006Cell wall
VIT_18s0001g08380	All	Berry	Stress	KNAT1_1	0.75081	15	1943	785	Homeobox protein knotted-1 like 1 (KNAT1)	N	vv60034HB
VIT_13s0064g00940	All	Berry	Stress	FRD3	0.74901	17	2427	661	ferric reductase defective 3	PM	vv50125Porters cat 66 to 94
VIT_07s0005g03530	All	Berry	Stress	LSH3	0.74648	19	266	43	Ribulose biphosphate carboxylase, large chain	N	vv10710Carbon fixation
VIT_01s0127g00210	All	Berry	Stress	KNAT6_1	0.74432	21	4597	1148	KNAT2 (knotted1-like homeobox gene 6)	N	vv60034HB
VIT_00s0515g00050	All	Berry	Stress	HT1	0.74975	23	909	470	Serine/threonine-protein kinase HT1	ND	ND
VIT_18s0072g01060	All	Berry	Stress	VND7	0.73541	26	348	184	Vascular related NAC-domain protein 7	N	vv60046NAC
VIT_02s0025g00870	All	Berry	Stress	No hit	0.73564	29	1281	3434	No hit	ND	ND
VIT_09s0002g09080	All	Berry	Stress	ZFP3	0.73183	30	200	684	Zinc finger protein 3	N	vv60017C2H2
VIT_17s0000g08030	All	Berry	Stress	SPDS1	0.7307	31	588	161	Spermidine synthase 2	EX C C/M CY CY/ER VM CYS/PM CYS/N CYS CY/P PM N/PM N	vv10220Urea cycle and metabolism of amino groups vv10271Methionine metabolism
VIT_00s0510g00030	All	Berry	Stress	PRXR4	0.74326	33	2838	337	Peroxidase 64	CY N ER ER/PM	vv10360Phenylalanine metabolism vv10680Methane metabolism
VIT_19s0090g00530	All	Berry	Stress	GGR	0.72786	35	75	564	Geranyl diphosphate synthase small subunit	M	vv10100Biosynthesis of steroids vv10900Terpenoid biosynthesis
VIT_12s0059g01010	All	Berry	Stress	CSLB04	0.72771	36	1195	268	Cellulose synthase CSLB04	PM	vv40006Cell wall

Table 3: Co-expression results for TBL44 from the VTCdb (Wong et al., 2013).

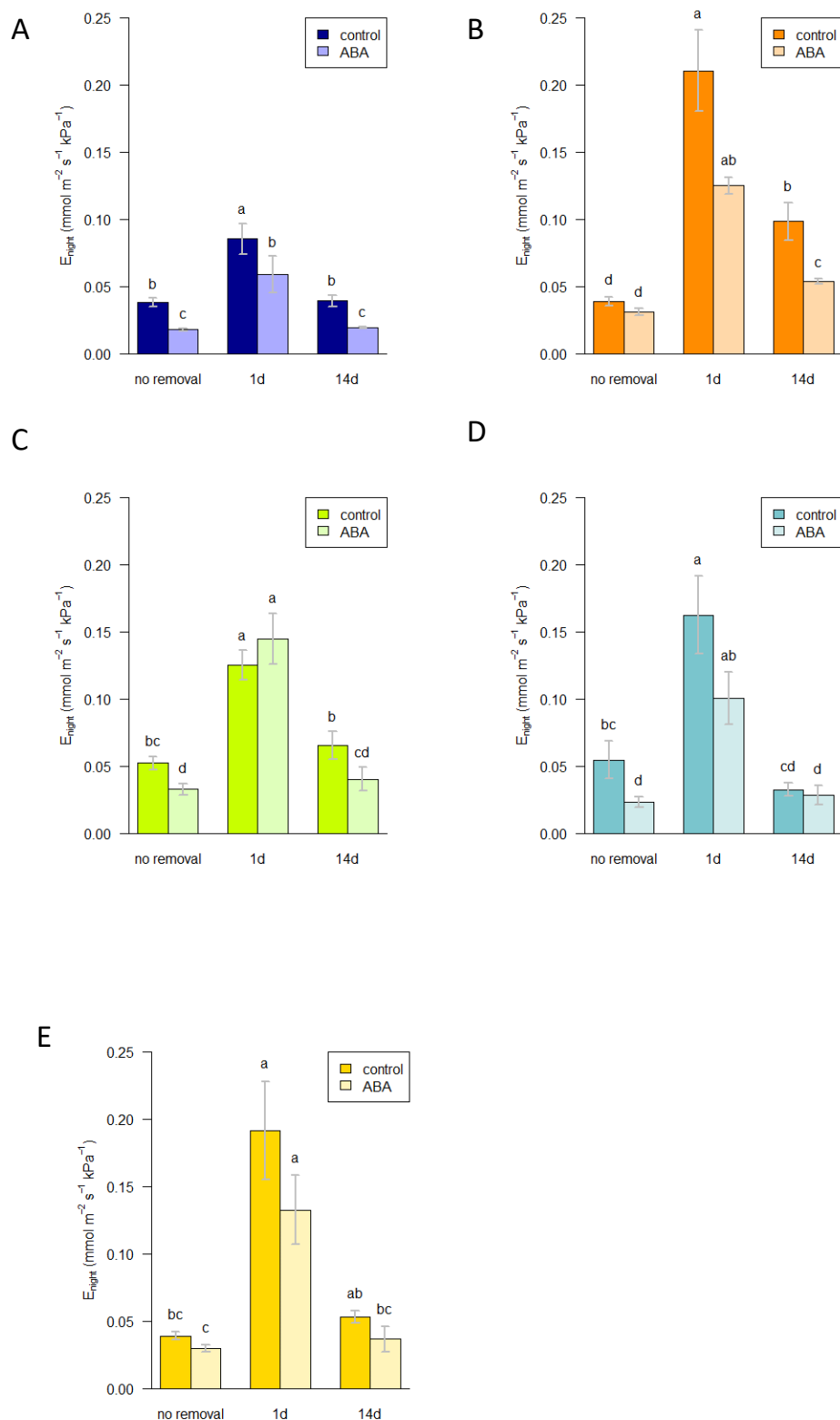
[Hide/show additional information on co-expressed genes](#)
[Show all co-expressed genes](#)

Target	Coex Query	Coex Query	Coex Query	Symbol	PCC	All	Berry	Stress	Functional annotation	Psort	Vitisnet molecular network
VIT_01s0011g06530	All	Berry	Stress	AKR	0.77949	7	49	216	Galacturonic acid reductase [Vitis vinifera]	N	vv40006Cell wall
VIT_11s0052g01240	All	Berry	Stress	DXS	0.77012	16	28	401	1-deoxy-D-xylulose-5-phosphate synthase	C	vv101008Biosynthesis of steroids
VIT_18s0001g01050	All	Berry	Stress	ZFC3HC4_112	0.75641	17	453	313	Zinc finger (C3HC4-type ring finger)	CY CY/P ER CY/N	vv60078Other zf-C3HC4
VIT_08s0007g07800	All	Berry	Stress	RABA4D	0.76566	23	13	188	RAB GTPase RABA4D	CY CY/ER CY/P C	ND
VIT_00s0445g00050	All	Berry	Stress	Unknown	0.75969	28	190	465	Unknown	C	ND
VIT_10s0042g01090	All	Berry	Stress	IRT1	0.74497	30	323	96	Iron regulated transporter	PM	vv50121Porters cat 1 to 6
VIT_00s0567g00030	All	Berry	Stress	Patatin	0.75521	30	103	33	Patatin	ND	ND
VIT_01s0011g06370	All	Berry	Stress	CAP13	0.76738	33	65	1901	Clathrin assembly protein 13	C	vv50110Protein coat
VIT_13s0019g03300	All	Berry	Stress	Glycosyl hydrolase family 17	0.76762	34	229	129	Glycosyl hydrolase family 17	VM	ND
VIT_12s0055g00820	All	Berry	Stress	scpl42	0.74201	36	364	7	Serine carboxypeptidase II	ER EX ER/VM CY	ND
VIT_00s0371g00110	All	Berry	Stress	RHGL	0.74188	38	318	287	Rhamnogalacturonate lyase	CY	vv40006Cell wall
VIT_01s0011g03300	All	Berry	Stress	ribosome	0.78651	41	344	141	Plastid-specific 30S ribosomal protein 3	C CY/N VM M M/PM CY/M ER EX	vv23010Ribosome
VIT_02s0025g00560	All	Berry	Stress	hydrolase	0.73934	43	42	688	Glycoside hydrolase, family 5	C	ND
VIT_05s0049g02260	All	Berry	Stress	MYB305	0.79034	48	314	197	Myb domain protein 305	N	vv60044MYB
VIT_03s0063g01830	All	Berry	Stress	CYP74A	0.73423	53	504	1642	AOS (allene oxide synthase)	C	vv10592alpha-Linolenic acid metabolism
VIT_01s0010g00460	All	Berry	Stress	transferase, transferring glycosyl groups	0.73028	56	625	406	Serine carboxypeptidase	CY C C/M P N	ND
VIT_09s0002g07960	All	Berry	Stress	No hit	0.75289	56	541	77	No hit	CY N	ND
VIT_15s0048g00590	All	Berry	Stress	BAG7	0.74253	58	203	19	BCL-2-associated athanogene 7 ATBAG7	CY CY/P C PM C/M EX	ND
VIT_10s0116g00300	All	Berry	Stress	HIN1-related	0.73775	58	474	951	Harpin-induced protein-related	M C/M	ND
VIT_00s0560g00010	All	Berry	Stress	Unknown	0.72849	60	854	522	Unknown	N CY	ND
VIT_07s0005g02320	All	Berry	Stress	ABCG21	0.73757	61	489	160	ABC transporter G member 21	ND	vv52010ABC transporters
VIT_15s0046g02600	All	Berry	Stress	Carboxyl-terminal peptidase	0.76364	62	277	402	Carboxyl-terminal peptidase	C GA EX M CY/ER CY/M VM ER ER/VM PM ER/PM CY/PM CY	ND
VIT_05s0051g00390	All	Berry	Stress	disease resistance-responsive	0.72657	62	84	452	Disease resistance-responsive	EX VM C/M M ER/VM C	ND
VIT_00s0496g00010	All	Berry	Stress	OPT1	0.76238	64	627	236	Oligopeptide transporter 1	C N	vv50125Porters cat 66 to 94
VIT_00s0211g00140	All	Berry	Stress	AGL21_6	0.72558	65	93	1397	MADS-box agamous-like 21	N CY/N M C/M CY C	vv60042MADS
VIT_14s0066g01980	All	Berry	Stress	matrixin	0.74861	67	588	867	Matrix metalloproteinase	EX C	ND
VIT_16s0050g01800	All	Berry	Stress	MFS	0.74982	68	224	671	Major Facilitator Superfamily MFS	ND	vv50121Porters cat 1 to 6
VIT_15s0107g00380	All	Berry	Stress	BHLHDOM_10	0.72484	69	25	59	Basic helix-loop-helix (bHLH) family	N	vv60011BHLH

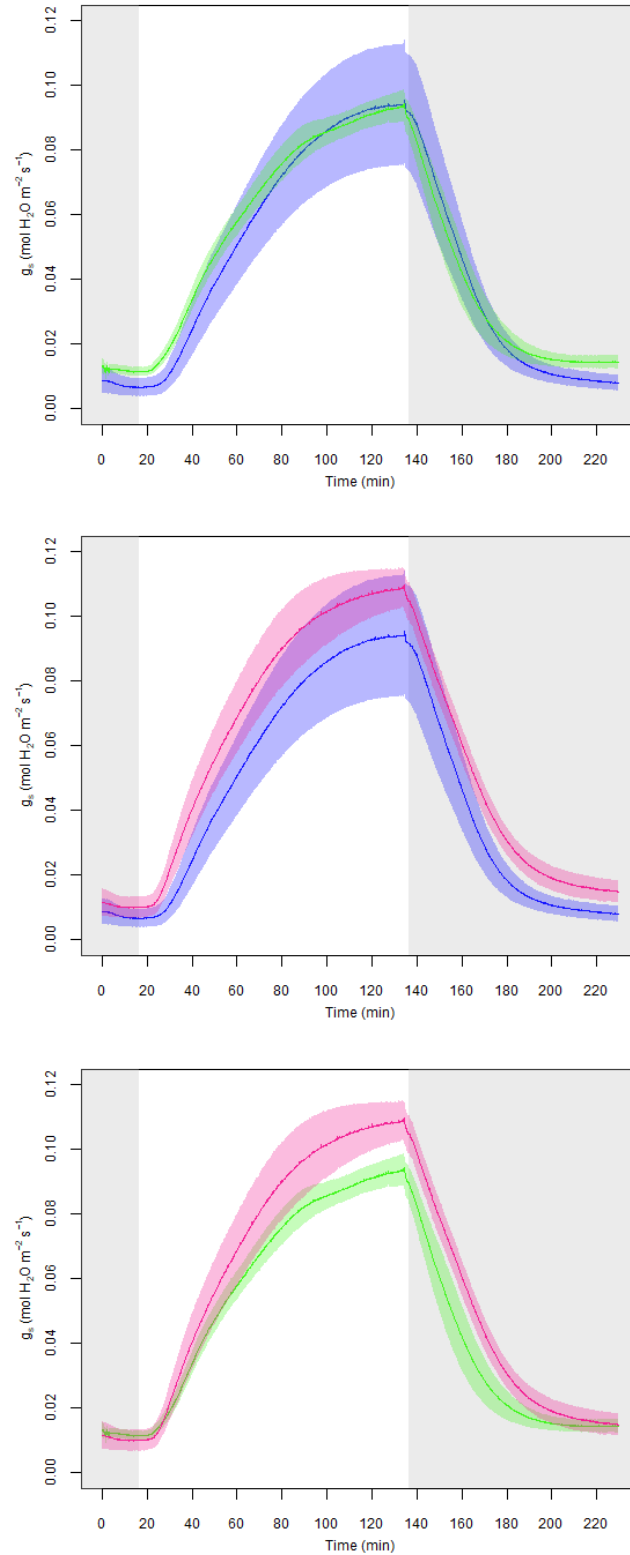
Table 4: Co-expression results for MYBF2 from the VTCdb (Wong et al., 2013).

[Hide/show additional information on co-expressed genes](#)
[Show all co-expressed genes](#)

Target	Coex Query	Coex Query	Coex Query	Symbol	PCC	All	Berry	Stress	Functional annotation	Psort	Vitisnet molecular network
VIT_17s0000g06410	All	Berry	Stress	MIXTAL2	0.80832	1	101	89	MYB transcription factor MIXTA-like 2	N	vv30009Flower development vv60044MYB
VIT_00s1333g00010	All	Berry	Stress	PHO1	0.78967	3	173	127	PHO1-like protein	PM	vv50125Porters cat 66 to 94
VIT_16s0050g02500	All	Berry	Stress	MUTE_1	0.78288	5	114	1054	Transcription factor MUTE	N	vv60011BHLH
VIT_01s0011g03230	All	Berry	Stress	AHBP1B_1	0.79353	7	157	676	Basic/Leu zipper protein HBP-1b	N	vv30011Jasmonate signaling vv60012BZIP
VIT_16s0098g00380	All	Berry	Stress	Wax synthase	0.7732	7	60	38	Wax synthase	CY CY/ER N CYS/N ER C CY/P CY/PM	vv11000Single reactions
VIT_06s0080g00290	All	Berry	Stress	TFL1	0.77267	9	462	1096	TFL1 (Terminal flower 1)	CY	vv30009Flower development
VIT_10s0116g00800	All	Berry	Stress	CYP77B1	0.76885	10	22	2433	CYP77A3	CY CY/ER PM	ND
VIT_08s0007g04080	All	Berry	Stress	CYP76C7	0.76688	12	343	260	CYPLXXVIA1	CY CY/ER CY/P N	ND
VIT_01s0011g02920	All	Berry	Stress	DICHOTOMA	0.76034	16	381	296	TCP family transcription factor DICHOTOMA	N GA CY/N	vv30009Flower development vv60061TCP
VIT_15s0046g01580	All	Berry	Stress	CHIB1	0.7626	17	175	1197	Acidic chitinase III	N PM CY/P ER/PM CY CYS/N P ER	vv10530Aminosugars metabolism
VIT_03s0038g02630	All	Berry	Stress	No hit	0.75796	20	201	403	No hit	N C/M M C	ND
VIT_00s0391g00030	All	Berry	Stress	Unknown	0.75064	30	146	748	Unknown	EX C	ND
VIT_03s0088g00680	All	Berry	Stress	PRP1	0.74679	34	633	1367	Pathogenesis-related protein 1 precursor (PRP 1)	C C/M ER	vv30011Jasmonate signaling vv34626Plant-pathogen interaction
VIT_01s0011g06430	All	Berry	Stress	AKR	0.75371	36	159	35	NADPH-dependent codeinone reductase	CY	vv10950Alkaloid biosynthesis I
VIT_07s0005g03250	All	Berry	Stress	No hit	0.75714	38	30	845	No hit	N C CYS/N N/PM CY	ND
VIT_19s0014g03220	All	Berry	Stress	EPF1	0.74784	42	75	879	Unknown protein	EX VM C	ND
VIT_15s0021g00180	All	Berry	Stress	RPP13	0.74052	45	801	472	RPP13 (recognition of peronospora parasitica 13)	ND	vv34626Plant-pathogen interaction
VIT_03s0063g01350	All	Berry	Stress	DOF5_8	0.73984	46	320	103	Dof zinc finger protein DOF5.8	N	vv60015C2C2-DOF
VIT_05s0094g00140	All	Berry	Stress	Protein binding protein	0.74455	47	449	673	Protein binding protein	ND	ND
VIT_11s0016g03970	All	Berry	Stress	UBC37	0.73707	52	649	54	Ubiquitin-conjugating enzyme E2 D/E	N CY/N C PM	vv24120Ubiquitin mediated proteolysis vv24141Protein processing in endoplasmic reticulum
VIT_08s0032g00640	All	Berry	Stress	Unknown	0.73671	53	774	5	Unknown	CY N CY/ER CYS/N N/PM EX C	ND
VIT_06s0004g01730	All	Berry	Stress	Unknown protein	0.75182	54	258	1310	Unknown protein	C ER/PM ER C/M N CY/N CY	ND
VIT_06s0004g03780	All	Berry	Stress	WOX3	0.74382	57	185	353	Wuschel-related homeobox 3 pressed flower	N CY/N	vv60034HB
VIT_09s0002g04200	All	Berry	Stress	No hit	0.73458	57	140	2096	No hit	C/M M C	ND



Supplementary figure 10: Effect of mechanical hair removal on transpiration values in different genotypes. Five leaves were genotype per treatment with or without 500 μ M ABA were peeled manually either one or 14 days before the transpiration assay. The following genotypes were tested: hairless Grenache (A), woolly Pinot Meunier (B), hairy Macabeu (C), low hair-dense Pinot Noir (D) and hairy Lledoner Pelut, the somatic hairy variant of Grenache (E).



Supplementary figure 11: Stomatal responses to light and darkness in three genotypes with contrasting prostrate hair density. The colours correspond to glabrous Grenache (blue), hairy Lledoner Pelut (pink) and hairy Syrah (green).

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Chapter 5

General Discussion

General Discussion

Plant evolution has successfully resulted in plants surviving in almost all environments existing on earth. A major adaptation of the early plants that colonized the land 500 Mya, was to adapt to lower water availability and dry air. Specialized organs have been developed to absorb (roots, Raven and Edwards, 2001), to transport (vascular tissue, Lucas et al., 2013) and to use water in photosynthetic organs (leaves, Harrison and Morris, 2018). Once developed in a relatively dry atmosphere, leaves need to avoid dehydration while at the same time maximizing gas entry of CO₂. Dehydration has been avoided by the development of a waxy layer surrounding the leaf called the cuticle (Kong et al., 2020; Renault et al., 2017), and CO₂ entry has been facilitated by pores in the cuticle called stomata (Berry et al., 2010; Chater et al., 2017). Opening of these pores for CO₂ entry inevitably results in water loss but the active regulation of its aperture avoids unnecessary or unwanted losses. For example, stomata close when there is no more need for CO₂ (high CO₂ concentration, low light) or in conditions of low soil water availability or dry air. Understanding the stomatal regulation of transpiration is an expanding research field as it can help selecting plants with improved water use (Masle et al., 2005; Papanatsiou et al., 2016), which is nowadays extremely important to battle water scarcity in agriculture.

The work presented in this PhD aims to understand the regulation of transpiration at night to improve plant water use. Nighttime transpiration is a widely existing phenomenon in nature and rates can be as high as 30% of the rates observed in the daytime (Barbour and Buckley, 2007; Caird et al., 2007a, 2007b; Dawson et al., 2007). The possible beneficial effects of nighttime transpiration on nutrient uptake and growth are contested (Auchincloss et al., 2014; Christman et al., 2009; Tanner and Beevers, 2001), and therefore these water losses are generally seen as unnecessary. A recent study showed that genetic variation exists for nighttime transpiration in grapevine which reduces transpiration efficiency (Coupel-Ledru et al., 2016). Stomata are suspected to cause nighttime transpiration by incomplete closure (Coupel-Ledru et al., 2016; Schwabe, 1952) or by reopening during the night (Caird et al., 2007a). A gradual increase in stomatal conductance at night has been observed in many species in natural or controlled conditions (Donovan et al., 2003; Leymarie et al., 1998, 1999; Rogiers et al., 2009; Yoo et al., 2009). This stomatal behaviour at night may potentially increase nighttime transpiration, but to what extent and by which signals is yet to be uncovered.

Development of a protocol to monitor diel stomatal dynamics

We developed a high-throughput phenotyping pipeline to monitor diel transpiration patterns in controlled conditions. A climate chamber equipped with a mobile scale was used to measure weight losses twice an hour during day and night. Gravimetric data was automatically cleaned for potential outliers by the developed data analysis pipeline, followed by the estimation of temporal transpiration patterns using a floating linear regression model. The protocol was applied to *Arabidopsis thaliana*, a model species in plant science and grapevine (*Vitis vinifera*), an important fruit crop which is particularly sensitive to the drier conditions which accompany the climate change (van Leeuwen et al., 2019; Moriondo et al., 2013). A candidate approach aimed to investigate the potential influence of starch synthesis and degradation on diel stomatal dynamics by using mutants of *Arabidopsis thaliana*. An unbiased approach was carried out by screening genetic variation in grapevine to evaluate the impact of nocturnal stomatal dynamics onto nighttime transpiration as well as to identify the genetic determinants of diel stomatal movements. In grapevine, the use of detached leaves possess several advantages compared to whole-plants: little space is needed allowing more replicates to be tested in a shorter time-frame, variation induced by long-distance signals is homogenised between genotypes (Jia and Zhang, 2008) and differences in transpiration rates can be directly contributed to stomatal behaviour whereas growth, leaf movements and tissue rehydration complicate this in whole-plant gravimetric experiments. Root-borne chemical signals such as ABA (Davies and Zhang, 1991) or pH

variations in xylem sap (Davies et al., 2001) are known to modulate stomatal behaviour and were homogenised by using a single solution with high-similarity to xylem sap as observed in the field. Most importantly, the transpiration dynamic of detached grapevine leaves matched to the dynamic of whole-plants without symptoms of stress during 24 hours at least. Total transpiration levels of whole-plants were slightly higher than detached leaves which may be due to the contribution of older and younger leaves that have generally a higher transpiration (Hopper et al., 2014; Kane et al., 2020; Pantin et al., 2013).

The detached leaf protocol unavoidably induces the wounding of plant cells at the base of the petiole which could provoke stress responses. Wounding initiates a range of processes in the leaf such as those induced by leaf herbivory or biotic pathogens, which can include the reparation of wounded tissues to maximize survival. Wounding response is regulated by fatty acids of the jasmonate family which are produced in the chloroplast in the vicinity of the wound (Weber et al., 1997). These fatty acids trigger the synthesis of jasmonic acid (JA), an essential defence-related hormone that is transported systemically in the whole-plant to trigger responses upon wounding such as stomatal closure (Evans, 2003; Meza-Canales et al., 2017; Reymond et al., 2004). Systemic responses to wounding are likely to depend on the intensity and speed of signalling gated by transpiration and can be rapid: within one hour not only local but also systemic increases in plant hormones such as JA and stomatal-closing hormone ABA were observed in wounded *Nicotiana attenuata* plants, however this did not alter stomatal conductance in distant leaves after 4 hours (Meza-Canales et al., 2017). The transpiration of detached grapevine leaves did decrease in the afternoon but followed a similar pattern as whole-plant grapevines and *Arabidopsis thaliana* and thus is not likely to result from wounding-induced responses (Chapter 2). If it would be, this response is not strong enough to impair the light-induced stomatal opening the next day (Chapter 2). However, we cannot exclude the existence of genotype-dependent response to wounding, as mutants have been characterised showing a stronger jasmonic-acid defence response (Bonaventure et al., 2007). In our experiments, we considered that reversion of stomatal aperture from day to night conditions for a period of at least 24 h in most cases was indicative of minimal influence such wounding effects on transpiration.

Leaf excision may also induce the entry of air in the xylem vessels, known as cavitation, which blocks the water transport to the leaf resulting in dehydration and stomatal closure. During leaf collection cavitation was avoided by re-cutting the petiole while immersed in water. Cavitation may also have occurred later during the experiment by air bubbles in the artificial xylem sap (AXS). Initial experiments were carried out with degassed AXS, however when scaling up the number of leaves, we lacked a facility to produce the large volumes of degassed AXS. Cavitation of leaves before the onset of the night generally resulted in sharp decrease in transpiration during the day followed by an incomplete stomatal closure at the light/dark transition. All other leaves showed a strong response to the light/dark transition indicative of a normal functioning of stomata. Cavitation during the experiment was therefore easily detectable and was not a common phenomenon (less than 4%), but it was observed that leaves with a high transpiration in the chamber had a higher risk to cavitate. High transpiration rates can be genotype-dependent and may vary with the position in the nursery. The genotypic effect on transpiration levels after installation in the chamber can be confounded with the position in the nursery which were not randomised because of practical reasons for leaf collection and identification of genotypes in the nursery. Having all replicates of one genotype on a single position facilitated the choice of a representative leaf. However, spatial variation in environmental conditions within the nursery are expected to have influenced the variation in our detached leaf assays. Spatial variation during growth could potentially have arisen from differences in microclimate within and between the ranks, variation in the level of ferti-irrigation along the ranks and border effects i.e. plants on the ends of the rank which are more exposed to wind and light. Transpiration but also plant growth

are likely to be influenced by these factors. We tested the effect of weather conditions before leaf collection and orientation of the leaf in the nursery (east of west). Both air temperature and leaf orientation in the nursery had a significant effect on transpiration levels in the chamber. However, both factors were not randomised in time: air temperature had a tendency to increase during the experiment while leaf orientation was involuntarily switched halfway the experiment as leaves of the west-side were burned by a heatwave (Chapter 2). These non-random effects were carefully tested in the mixed model to avoid the elimination of genetic variation. Spatial effects of microclimate on transpiration were also evidenced after heatmap analysis of the randomised transpiration levels in the climate chamber (Chapter 2). Heatmap analysis revealed a gradient in transpiration, almost doubling from one extreme position to the other, that differed between day and night. These gradients may be due to spatial variation in light intensity and/or humidification of the chamber. Due to randomisation of leaves in the climate chamber, we were able to investigate the effects of spatial variation on transpiration. The results of this analysis can help future experimental design and climate regulation, but most importantly enabled us to correct for these effects which improved the power of genetic analysis tremendously. Similarly, an effort should be undertaken to randomise plants in the nursery for future detached leaf assays allowing to correct for outdoor spatial variation and facilitating the genetic analysis by improving heritability. Nevertheless, our protocol was able to detect significant variation between genotypes with a heritability around 0.3 for transpiration rates allowing us to identify the genetic controllers of daytime and nighttime transpiration.

Nighttime stomatal reopening

Gradual increases in transpiration rates during the night were observed in *Arabidopsis thaliana* but to a lesser extent in grapevine. *Arabidopsis* increased transpiration (up to 30%) which could only be partly explained by growth (8%) (Chapter 2). It is not likely that nighttime increase in transpiration in *Arabidopsis* is caused by vertical leaf movements as leaves are expected to move from an upward to downward position during the night (Flood et al., 2016) and thus a gradual nighttime decrease in transpiration would be expected. A gradual increase in nighttime transpiration could still be observed after correction for the specific leaf expansion of rosette area at night. This suggests that stomata did reopen during the night, a phenomenon that has been widely observed in outdoor conditions but not always in indoor conditions (Andrés et al., 2014).

In outdoor conditions, the opening of stomata at night has been proposed to be a stomatal response to the gradual decrease in VPD during the night. This could be due to the progressive rehydration of tissues at night thereby increasing guard cell turgor and subsequent reopening. However, this was expected to play a minor role on stomatal behaviour in our detached leaf assays as they were immersed in the solution for at least 10 hours before the onset of the night. Moreover, during an internship at our laboratory (Guiguitant, 2017), experiments were performed where VPD was manipulated in different ways during the night and showed that stomata were irresponsive to VPD changes at night. Another hypothesis which could explain nighttime stomatal reopening is the response of stomata to high internal CO₂ levels produced by respiration (Even et al., 2018; Fricke, 2019) or diminishing ABA concentrations at night regulated by the circadian clock (Pokhilko et al., 2013).

It is puzzling that stomatal reopening was observed to a much smaller extent in grapevine. In our comparison experiment between whole-plants and detached leaves, we observed the small increase in stomatal opening at night only in whole-plants. However, depending on the day, genotype, and probably growing conditions of the leaf, reopening patterns could also be observed in detached leaves. The observation that detached leaves could show reopening in controlled conditions suggests that endogenous, leaf-level signals control the stomatal reopening at night. In this PhD study, we could not identify the factors causing such variation in stomatal behaviour. Seasonal variability in stomatal

reopening at night has been observed before in *Chrysotamnus* (Donovan et al., 2003) and it is expected that the age of the leaf also plays an important role. Although grapevine leaves were sampled at similar stage of development in our study, assays lasted several weeks with slight differences in plant age between the beginning and the end of experiments. This could have caused slight drifts in leaf physiology and stomatal behaviour.

The daytime conditions may also influence nighttime stomatal apertures. For example, increased photosynthesis levels in *Vicia faba* resulted in higher stomatal opening in the following night (Easlon and Richards, 2009). Starch metabolism appears as a good candidate involved in this response. The more it accumulates during the day, the more sugars can be derived from starch breakdown during the following night which may diffuse to the apoplast of guard cells to induce stomatal opening (Stadler et al., 2003).

Stomatal reopening at the end of the night may also be thought as an adaptation of the leaf in order to open faster at the start of the day. However, in sunflower this did not lead to a higher photosynthesis (Auchincloss et al., 2014). We observed a high variability in the level of nighttime increases in transpiration in *Arabidopsis* mutants impaired in the starch breakdown or synthesis. However, as growth may have influenced these transpiration patterns we cannot confirm if the lower increase in transpiration at night was caused by an absence of stomatal opening or a smaller growth rate. Modelling approaches in the future will help to understand to what extent the growth patterns may influence the transpiration dynamic at night in these mutants. This implies that growth patterns should be carefully monitored during the diel cycle in order to be able to draw conclusions from gravimetric data about stomatal movements in a fast-growing plant as *Arabidopsis*.

Origins of the genetic variation in nighttime transpiration: cuticle, leaky stomata or trichomes?

The large genetic variation in the levels of nighttime transpiration which was observed in the biparental population for grapevine (Coupel-Ledru et al., 2016) was confirmed in our study on a diversity panel. The origins of this variation have been partly enlightened by our genetic analysis together with our exploration of some morpho-physiological candidates. A huge difference was also observed in nighttime transpiration levels between *Arabidopsis thaliana* and *Vitis vinifera*. In both species, transpiration rate was reduced to a similar extent in response to darkness. First, it is discussed whether the residual transpiration may be due to incomplete stomatal closure, so-called “leaky-stomata”, or to the direct water losses from the epidermis through the cuticle. We found that nighttime transpiration in grapevine was controlled by a QTL that also controlled the density of prostrate trichomes and cuticular structures. Prostrate trichomes in grapevine are known to protect against pathogens (Kono et al., 2018), and to reduce transpiration by increasing the boundary layer resistance to transpiration from the leaf to the air (Bickford, 2016; Yoo et al., 2009). By contrast, in our study, prostrate trichomes correlated positively with the level of nighttime transpiration in grapevine. Trichomes may also increase the evaporative area of the leaf, but this is unlikely as prostrate trichomes are thought to be completely dry and so not able to convey water out the leaf (Gago et al., 2016). The correlation that we observed between prostrate trichomes and nighttime transpiration could therefore be indirect and may involve a third actor, genetically linked to trichome development but with a more direct role on transpiration, such as stomata. However, the difference in nighttime transpiration between glabrous and hairy leaf cultivars was conserved after ABA treatment, suggesting that it may not be related to incomplete stomatal closure or stomatal density. Indeed, no correlation was observed between the density of trichomes and stomata, but it has to be mentioned that phenotyping of stomata was complicated by the tortuous epidermal surface of some genotypes.

Epidermal structures were observed on the imprints which had close similarity with cuticular ridges described before in the *Vitis* genus (Ickert-Bond et al., 2018; Konlechner and Sauer, 2016). These ridges are also observed in other species on all shoot organs (Casado and Heredia, 2001; Khan et al., 2018; Segado et al., 2016), and are mostly described for their high abundance on flowers which is thought to produce a higher diffraction of light, leading to more intense and pure colours to attract pollinators (Kourounioti et al., 2013). Evidence is accumulating that ridges are caused by folding of the cuticle as a result of an imbalance between the secretion of cuticle and epidermal cell expansion (Hong et al., 2017; Jeffree, 2006; Martens, 1933). This hypothesis is supported by the absence of ridges in flowers of *Arabidopsis* mutants disrupted in cuticle biosynthesis (Li-Beisson et al., 2009; Mazurek et al., 2017; Panikashvili et al., 2011; Shi et al., 2011). Surprisingly, in these mutants the absence of ridges was associated with a higher permeability of the epidermis. However, the composition of the cuticle also changed in these mutants with possible implications on the permeability. A better comparison can be made with the SHINE mutant: this is a transcription factor regulating the wax production of the cuticle (Aharoni et al., 2004). Mutating this gene resulted in a six-fold increase in total cuticular wax level, produced nanoridges on cauline leaves, and showed permeability to water. It is concluded that ridges may increase transpiration by a different composition of the cuticle or by increasing the surface area of the leaf by a factor of 2 to 3, potentially increasing transpiration (Segado et al., 2016).

The role of the cuticle in controlling nighttime transpiration is supported by several observations. In maize, nighttime transpiration was shown to be genetically controlled by putative cuticular genes (Lin et al., 2020). In our study, we found that ABA reduced nighttime transpiration in *Arabidopsis* by only 20%, suggesting that the residual transpiration is mainly due to cuticular transpiration. The role of stomata in controlling transpiration at night was also ruled out in another study, where only slight, non-significant decreases in nighttime stomatal conductance were observed after increasing CO₂ concentrations whereas it was expected to close stomata (Costa et al., 2015). Interestingly, mutants with high nighttime transpiration in this latter study retained their higher transpiration levels after a similar induction of stomatal closure by ABA and CO₂ and showed a higher daytime transpiration. This may suggest that those mutants are affected in cuticular development resulting in a higher transpiration rate whatever the conditions, specifically after stomatal closure by darkness and darkness combined with high CO₂ treatment.

In our study, ABA treatment in grapevine leaves did reduce transpiration at night by ~50%, indicating that stomata were not completely closed at night. If ABA caused full stomatal closure (sealing the pore) in both detached grapevine and *Arabidopsis* leaves, the residual transpiration is caused by water transport through the cuticle. This would imply that cuticular permeability is 20 x higher in *Arabidopsis* compared to grapevine. Alternatively, we may question the efficiency of ABA to induce complete stomatal closure, possibly leaving room for genetic variation in stomatal 'leakiness' within our grapevine population or between grapevine and *Arabidopsis*. Leaky stomata may be the result of a variable loss of sensitivity to ABA leading to variable incomplete stomatal closure. Genetic variation in nighttime transpiration could also combine leaky stomata with genetic variation in stomatal density without necessary variation in stomatal leakiness. Regardless of this distinction, the relative importance of water loss at night through leaky stomata is supported by a positive correlation between stomatal density and minimum conductance in sorghum (Muchow and Sinclair, 1989). However, the setting up of stomatal density may be linked to cuticle development (Aharoni et al., 2004), resulting in a correlation between both traits and making difficult to unravel which one control transpiration. Some studies found that water losses during desiccation mainly occurred on the side of the leaf bearing stomata in hypostomatous species (Brodribb et al., 2014). This was also observed in *Hedera helix* but was attributed to the much higher permeability of the cuticle on the leaf side bearing stomata (Šantrůček et al., 2004). Thus, at this point we cannot know if the difference in nighttime conductance

between *Arabidopsis* and grapevine arises from difference in leaky stomata or/and in cuticle permeability.

Yet, stomata are thought to be the main actor controlling water losses at night by incomplete stomatal closure (Caird et al., 2007a). This conclusion has been drawn based upon the large differences observed between nighttime leaf conductance and after dry-down, which is the remaining conductance after dry-down being the most used technique to estimate the cuticular conductance (Borel et al., 2001; Rawson and Clarke, 1988). The dry-down technique consists out of leaf drying on the bench while monitoring leaf conductance with gas-exchange measurements or a scale. At the start of the experiment, water losses are high due to open stomata and after some-time this is strongly reduced, likely due to stomatal closure. At this time point, the leaf minimal conductance, assimilated to cuticular conductance, is measured. However at this moment it is likely that the epidermis also, including the cuticle itself, has dried out which has been shown to limit cuticular transpiration (Boyer et al., 1997; Kerstiens, 1995; Meidner, 1986; Tominaga et al., 2020). If so, cuticular conductance of a non-dehydrated leaf is underestimated with this technique thereby overestimating the stomatal contribution to nighttime transpiration. This widely used but not so robust protocol to measure cuticular conductance shows the difficulty to separate stomatal and cuticular transpiration. Gas-exchange analysers are not able to distinguish between both conductances and measures whole leaf water loss but confusingly, the output is systematically called stomatal conductance as if the cuticular contribution was negligible. We should rather call it total leaf conductance to avoid misinterpretations of the data as the epidermis may also contribute to the water loss depending on its hydration state. Stomatal opening *sensu stricto* may only be known by microscopic analysis which can be done in situ with microscope (Kaiser and Kappen, 1997). So the impact of the cuticle on transpiration may have been underestimated based on the methodological difficulty to separate both stomatal and cuticular conductances. Recent research addresses this problem and proposes new method to estimate the part of cuticular transport. Surprising the authors themselves, up to 20% of total transpiration was regulated by the cuticle in *Helianthus annuus* leaves (Hanson et al., 2016).

Cuticular development is dependent on growing conditions and may partly explain the difference between *Arabidopsis* and grapevine nighttime transpiration. Cuticle production is highly dependent on environmental factors such as light, humidity and temperature (Jenks and Ashworth, 1998). Plants grown outside are thought to experience more environmental fluctuations and to produce a thicker cuticle than plants grown in controlled conditions. This may explain why we found a lower average nighttime transpiration on outdoors-grown detached leaves, $0.04 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, than previously reported in whole-grapevines grown in a greenhouse (higher humidity levels), $0.08 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (Coupel-Ledru et al., 2016). The difference in growing conditions may also explain why different SNPs were found for nighttime transpiration in cross between grapevine cultivars Syrah and Grenache compared to our association panel, while the Syrah and Grenache exhibit allelic variation at this locus (Chapter 4). Moreover, the effect of the environment seems to be genotype dependent. For example, Grenache transpired less at night than Syrah in our detached leaf assays, two greenhouse experiments (Coupel-Ledru et al., 2016) and in the field (Rogiers et al., 2009) but in experiments performed during an internship at LEPSE (Guiguitant, 2017) greenhouse-grown Grenache transpired more than Syrah. Plants in the greenhouse or laboratory conditions are known to have a different morphology and a faster growth rate (Poorter et al., 2016). Growing in the greenhouse may require a smaller investment in the cuticle to protect against extreme temperatures and dehydration. Moreover, the major compound of the cuticle, lipids, are the most expensive compound to produce for the plant (Poorter and Villar, 1997). So, from an energy-efficient perspective, plants investing less in cuticular development will save energy for growth. This may be an important reason why plants show a high plasticity in the production of the cuticle. Overall, comparisons across studies should pay attention to

the growth conditions of the plant material which may induce some variations in the respective roles of the physiological traits acting on transpiration.

Origins of the genetic variation in stomata leakiness?

The stomatal response to darkness did not show a high plasticity. We observed that in all conditions, on detached and whole-plants, stomata responded to the day and night regime. The main reason of stomatal closure at night is thought to save excessive and inefficient water losses. However, plants grown in well-watered conditions as *Arabidopsis* in our study showed a similar amplitude of decrease in transpiration to grapevine, but this did not prevent large water losses in *Arabidopsis*. Even barley plants grown hydroponically strongly close stomata at night (Knipfer and Fricke, 2011). Stomatal closure at night in the absence of a risk to dehydrate was also questioned by F. Darwin (1898). The response to darkness may be a highly conserved response among plants, except those having a crassulacean acid metabolism (CAM), resulting from an evolutionary origin. Indeed, saving water was an important selection pressure in plant evolution as early plants needed to adapt to drier conditions when colonizing the land and so stomatal control may be of high importance. As such, the angiosperms have developed a much more elaborated system to control stomatal aperture than ferns, which allowed the former to colonize drier habitats. Angiosperms invented a complex network of signalling cascades and metabolites to control stomatal opening (Brodribb and McAdam, 2011; Doi et al., 2006; McAdam and Brodribb, 2012a, 2012b). Interestingly, ferns still respond to darkness (Doi et al., 2006; McAdam and Brodribb, 2012b). This suggests that stomata, which have a single evolutionary origin (Raven, 2002), have acquired very soon the ability to close in response to darkness. Apparently, the dark-induced stomatal closure may provide other benefits than only saving water, but what?

Likewise an important function of the cuticle, stomata are also a physical barrier against parasites. Therefore, a reason to close stomata at night could be to avoid the entry of pathogens. Many bacteria need high-humidity conditions to move on the leaf surface and more importantly need open stomata to create infection and proliferate. At night, the humidity conditions are favourable for bacterial infection. Bacteria *Pseudomonas syringae* even developed a phytotoxin that opens stomata at night called coronatine which is essential for successful proliferation of bacteria (Panchal et al., 2016). Coronatine influences the hormonal balance between jasmonic acid (JA) and salicylic acid (SA) (Panchal and Melotto, 2017). Moreover, stomatal clock mutants who do not close well at night have higher infection rates by pathogens (Melotto et al., 2017). The grapevine ‘bacterial inflorescence rot’ disease is caused by *Pseudomonas syringae* and distorts stomatal opening in order to enter and create infection (Whitelaw-Weckert et al., 2011). Not only bacteria need to enter through the stomata, but also oomycetes such as downy mildew (in grapevine known as “mildiou”, *Plasmopara viticola*) enter via stomata and increase stomatal opening in the dark (Allègre et al., 2007). Other important pathogens of grapevine, such as the fungi powdery mildew (in french “oidium”, *Uromyces necator*) and *Botrytis cinerea* penetrate directly through the cuticle by the formation of an appressorium which pressurises a hole into the cuticle (Armijo et al., 2016; Grimmer et al., 2012). Plants have developed specialized receptors that detect microbe-related elicitors on the epidermis which trigger defence mechanisms such as stomatal closure (Melotto et al., 2006). This helps the plant to respond only to pathogens when present on the leaf. Next to reactive responses of the plants against pathogens, stomatal closure at night may be a preventive strategy to avoid pathogen entry. So, closing the pores in absence of light may be advantageous to create an extra defence barrier against pathogens. Next, stomatal closure may be also beneficial for the plant in regard to the energy needed for stomatal opening.

Stomatal closure at night: induced by dark conditions or simple return to resting state in absence of light?

It is more likely that the stomatal closure in response to darkness is the inevitable result of absence of light which is a fundamental requirement for stomata to open. Red light is thought to induce stomatal opening via photosynthesis in the guard cells and the mesophyll, while blue light activates a plasma membrane H^+ ATPase that uses the energy from ATP hydrolysis to export H^+ out of the guard cells (Schroeder et al., 1987). Recently it was shown that also red light induces the activation of the H^+ ATPase (Ando and Kinoshita, 2018, 2019). The activation of the H^+ ATPase by especially blue light is known as the most effective way to open stomata in response to light (Kinoshita and Hayashi, 2011) and may be involved in the return to closed state. Blue light irradiation of guard cells activates blue light-photoreceptor protein kinases, called phototropins (PHOT1 and PHOT2 (Kinoshita et al., 2001)), through autophosphorylation and initiate signalling for stomatal opening (Inoue and Kinoshita, 2017). The activated phototropins phosphorylate the protein kinase BLUE LIGHT SIGNALING1 (BLUS1) (Takemiya and Shimazaki, 2016), and BLUS1 generates a signal to activate the H^+ -ATPase pump (mainly AHA1), in guard cells through another phosphorylation of a 14-3-3 protein (Cotelle and Leonhardt, 2016; Shimazaki et al., 2007). H^+ efflux results in the hyperpolarization of the membrane and activates the influx of K^+ and anions Cl^- , nitrate and malate (Kim et al., 2010; Lebaudy et al., 2008). The accumulating K^+ and Cl^- are directly transported into the vacuole via tonoplast transporters (De Angeli et al., 2013). The accumulation of these ions in the guard cells decreases the water potential, leading to water uptake and turgor increase (Inoue and Kinoshita, 2017). The light-activated stomatal opening is influenced by the level of red light (Shimazaki et al., 2007), signals of the photoperiodic flowering pathway (Kinoshita et al., 2011), the hormone ABA (Inoue and Kinoshita, 2017) and time of the day (circadian clock, Hubbard and Webb, 2011). So, stomatal opening is essentially activated by light-responses guided by phototropins.

In order to open stomata in response to light, ATP is necessary to activate the H^+ ATPases. The origin of the ATP or energy for stomatal opening is at least partly independent on the photosynthetic products of the mesophyll, as albino patches in the leaf also exhibit stomatal opening in response to light (Roelfsema et al., 2006). If ATP supply from the light-reactions in the chloroplasts of guard cells is impaired, stomata display attenuated stomatal opening in response to light (McLachlan et al., 2016; Wang et al., 2014a). Providing the ATP for the H^+ ATPase may be one of the important functions of guard cells chloroplasts. Interestingly, blocking an enzyme in the ATP absorbing Calvin cycle to produce sugars from CO_2 , increased stomatal opening in response to light (Lawson et al., 2008). This may be caused by a higher availability of ATP and NADPH for the proton pumps due to a lower consumption of ATP by the Calvin cycle. ATP is important to keep turgor in the guard cells by avoiding depolarization of the membrane resulting in solute and water efflux. Indeed, when impaired in solute efflux, stomata close more slowly (Meyer et al., 2010) or have a continuous stomatal opening (Sierla et al., 2018). At night, chloroplasts will produce less ATP, resulting in a lower activity of the H^+ ATPase proton pumps and closing of stomata. Indeed, even when overexpressing H^+ ATPases (AHA1 or AHA2), at night stomata closed (Merlot et al., 2007; Sierla et al., 2018; Wang et al., 2014b). Although ABA is expected to gate stomatal closure in response to darkness, mutants with low sensitivity to ABA such as *abi1-1* or *abi2-2* (Leymarie et al., 1998; Merilo et al., 2013; Webb and Hetherington, 1997) or *pyr/pcar* or *ost1-1* and *ost1-2* (Merilo et al., 2013; Mustilli et al., 2002) still showed stomatal closure in response to darkness. This shows that the response of stomatal closure to the dark is to a small extent gated by ABA and mainly by light, either via lower ATP supply of the chloroplasts or by the deactivation of the phototropins by light. The light-dependent response to darkness may also involve specific changes in actin dynamics not used by ABA to close stomata (Isner et al., 2017). So, stomatal closure in response to darkness may be the result of an energy saving mechanism back to its resting state.

The resting state of stomata decides the contribution of leaky stomata to nighttime transpiration. ABA signalling and concentration are expected to be important regulators for the resting state: ABA-insensitive mutants have generally a higher transpiration during the night and day (Kollist et al., 2014; Leymarie et al., 1998). ABA accumulation, production and signalling are known to be dependent on the growth conditions. Especially when plants are grown in high humidity conditions, stomata are known to be less responsive to extreme conditions such as low humidity which is major problem for the production of cut flowers (Fanourakis et al., 2016). The sensitivity of varieties to extreme environmental conditions has been assigned to lower levels of ABA and stomatal irresponsiveness. To battle this problem, growers try to increase the levels of ABA in cultivars by partially drying of the roots, grafting on a rootstock producing higher ABA levels or changing environmental conditions by having a lower VPD, blue light treatments (Terfa et al., 2020) or inducing mechanical stress (Fanourakis et al., 2016). Generally, plants experience more stress outdoors due to wind, high light, and extreme fluctuations in temperature and humidity. It is expected that plants outdoors produce more ABA due to high evaporative demand and fluctuations in soil water availability, which influences basal stomatal opening during the day and the night. The expected lower levels of ABA in *Arabidopsis thaliana* grown in laboratory conditions may therefore influence the basal opening levels of stomata during the day and also at night. Thus elevated day and nighttime transpiration in *Arabidopsis thaliana* may partly be explained by the resting state of stomata which is more open in laboratory conditions. It is concluded that higher stomatal opening at night is not the result of incomplete stomatal closure but rather a different resting state of stomata (Figure 1).

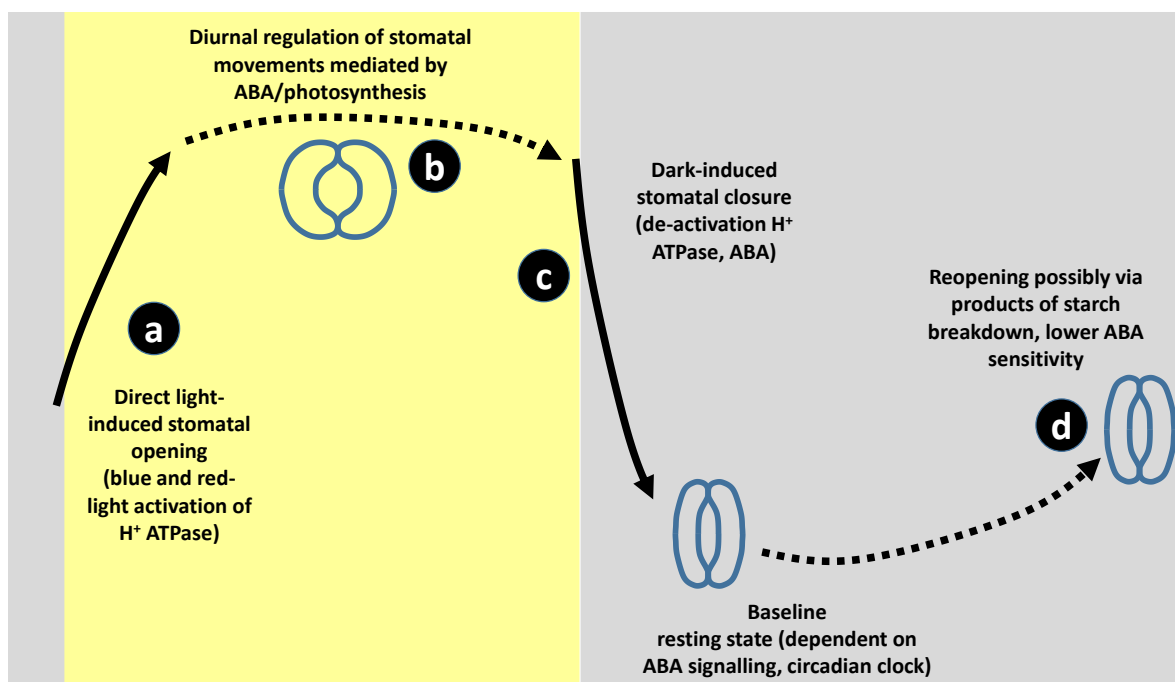


Figure 1: Schematic representation of processes controlling diel stomatal movements. Light-induced reopening is mainly mediated by blue light and a lower extent by red light activation of both H^+ ATPases and starch degradation (a). Further reopening and closure patterns during the day can be mediated by photosynthesis-derived signals including sugars or ATP, or by ABA signalling of the guard cells depending on the circadian clock and growing conditions (b). Stomata rapidly close at night mainly by the deactivation of the light-induced opening process and to a lesser extent ABA. The opening of stomata in its baseline resting state is dependent on ABA concentrations and/or sensitivity depending on e.g. the circadian clock and growing conditions. Nighttime stomatal reopening can be observed

during the night as a result of lower ABA sensitivity of the guard cells or induced by the products of starch degradation (d).

Nighttime transpiration: from bioassay in climate chamber to the field

By using the detached leaf protocol, we identified a genomic region controlling nighttime transpiration in grapevine. Moreover, multiple epidermal structures such as prostrate trichomes and cuticular ridges are controlled by the same region. Analysis of the candidate genes in the region together with exploration of expression databases pointed to MYBF2 as an exciting candidate to control nighttime transpiration in grapevine. Although this gene has close homology with other flavonol R2R3 transcription factors, it lacks the shared motifs important for flavonol protein regulation possibly suggesting a different function of the gene (Czemmel et al., 2009; Stracke et al., 2001). We propose that MYBF2 has been subject to neofunctionalization with new functions related to epidermal trichomes development and concomitant impact on night transpiration. In agreement with our hypothesis of neofunctionalization, trichome mutants altered in MYB sequence often show defects in pigmentation (Airoidi et al., 2019), and are thought to originate from duplications of a single anthocyanin synthesis gene (Serna and Martin, 2006). Interestingly, MYBF2 is expressed during leaf development at bud burst (Fasoli et al., 2012), and is co-expressed with genes involved in epidermal development such as MUTE and Wax synthase (Wong et al., 2013) and with MIXTA3, one of the MIXTA genes known to play a role in cuticle and trichome development at the same time in *Arabidopsis*, tobacco and *Antirrhinum* (Galdon-Armero et al., 2020; Glover et al., 1998; Perez-Rodriguez, 2005). Moreover, MIXTA genes may also produce cotton fibers which have a similar appearance to prostrate trichomes found in grapevine leaves (Machado et al., 2009; Walford et al., 2011). The co-expression with MUTE suggest that the gene is expressed stomatal differentiation from the meristemoid (MacAlister et al., 2007). MUTE is also involved in hydathode development (Pillitteri et al., 2008), which is a structure that may potentially contribute to leaf transpiration but to what extent is currently unknown. Our expectation is that we found a single gene or a hub of genes controlling several morphological traits of the epidermis as well as genetic variation in night transpiration. At this moment, sequence analysis is performed to identify the polymorphism in our candidate gene MYBF2, leading to the production of trichomes and cuticular nanoridges.

Decreasing nighttime transpiration in grapevine cultivars may be an elegant way to improve the water use efficiency of grapevines. Our protocol used grapevine leaves grown outside which may be more representative of field conditions than leaves grown in the greenhouse. Moreover, transpiration levels of whole-plants outdoors were similar to those observed on detached leaves in the chamber. Our results indicate that nighttime transpiration can only contribute to a small part of total day transpiration. However, cuticle-related traits are expected also to influence water use in the daytime., indicating that by using the QTL detected for this trait, we can save water on the total diel cycle. Even more water could be saved provided that trichomes characteristics could be selected independently of the cuticle, as more trichomes are expected to decrease both water and CO₂ exchange in the daytime while thicker cuticle can save water without altering photosynthesis. Ideally, uncoupling of the trichomes and permeable cuticle can help to breed for the optimal combination of traits depending on the local conditions. Lower water losses at night may save soil water that can be used later in the dry season to ensure maturation and minimal yield. The effect of a variety with a low nighttime transpiration was modelled in a vineyard water balance study *in silico* that we published in the *Revue des Oenologues* (Figure 2). This study showed that, if more water is saved at night, it will be used during the day and may lead to a higher carbon fixation. This may not be at the advantage of the water-saving variety as long as water availability is maintained at high level. However, in the second half of the plant

cycle, during maturation, soil generally dries down to low levels, which limits stomatal opening in the daytime. Nighttime transpiration then represents an increasing part of the 24-h water losses, and a variety with reduced nighttime transpiration at this stage may offer a substantial advantage in postponing severe soil dehydration that blocks maturation. Overall, a gene or a combination of genes reducing nighttime transpiration may be beneficial in two aspects: postponement of severe soil drying and increase in water available for maintaining gas exchange.

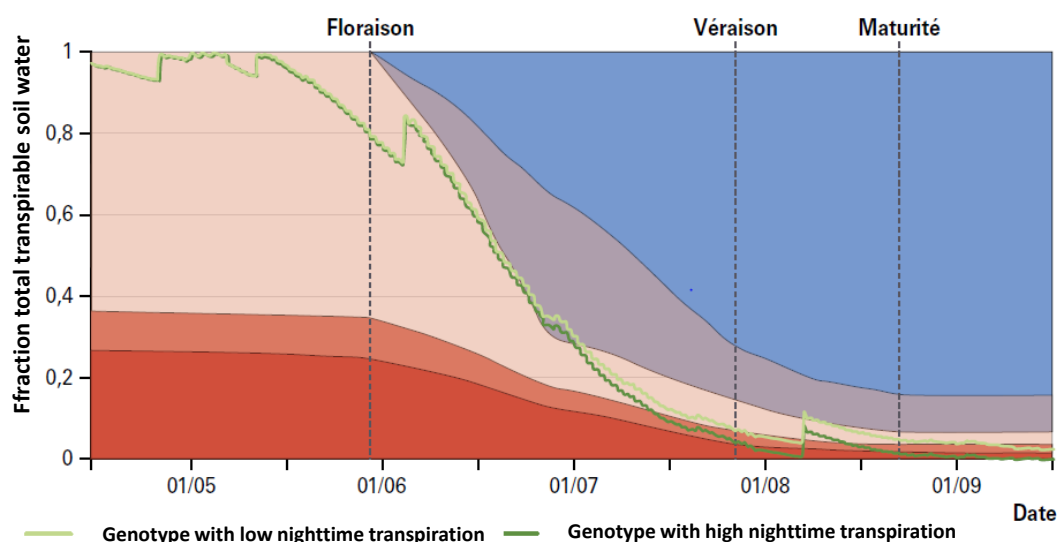


Figure 2: Simulation of the impact of nighttime transpiration on the water balance in the vineyard. Two genotypes are simulated: a water consuming genotype (23% of daytime transpiration, dark green) or water saving genotype (6% of daytime transpiration, olive colour). The simplified model of water balance in the vineyard (Lebon et al., 2003) uses hourly weather data of a season in Montpellier (France). The water stock in the soil (fraction transpirable soil water) is influenced by the evapotranspiration of the plant and soil, drainage and rainfall (represented by the peaks). The colors represent the preferred dryness of the soil during the cycle: blue is too wet, red is too dry. It can be observed that a genotype transpiring less during the night can avoid a water balance in the red zone which is likely to impact quality. The three vertical lines represent the important developmental stages of grapevine during the season: floraison (flowering), véraison (onset of ripening of grapes) and maturité (maturity). Figure adapted from Pantin et al. (2019).

Another major difference with the field is that grapevines are grafted onto a rootstock to avoid infection of phylloxera (*Daktulosphaira vitifoliae*). The rootstock has been shown to have significant effect on the shoot transpiration which is regulated by long-signalling chemical and hydraulic signals (Marguerit et al., 2012). This is probably linked to the production of ABA by the roots which is transported to the shoots and induces stomatal closure. It is not known to what extent rootstock may distort the results that we obtained on detached leaves. The correlation observed between nighttime transpiration of detached leaves and whole plants across a small set genotypes and for some environmental variation (ambient and elevated CO₂) suggests that most of the variation is conserved in the presence of a root system, but we did not vary the genotype of the root part, which remains to be explored.

In our study we did not see a strong correlation between daytime and nighttime transpiration as was seen before. This can be caused by the different growing conditions and protocols used to measure transpiration (detached leaves – whole-plant). Breeding for a cuticular trait may be advantageous

because of the low impact on daytime gas exchange and on photosynthesis. However, the production of lipids may require more energy of the plant. A cost-benefit balance should be drawn up to know in what conditions we need plants that exhibit a lower cuticular conductance. The impact of a cuticular based trait to reduce transpiration during the night may be of major interest in periods of drought-induced stomatal closure, avoiding leaf dehydration. If this trait was important for drought tolerance, it is expected that domestication may have selected grapevine genotypes with a more or less permeable cuticle. The pleiotropic effect of the locus on prostrate trichomes development may also have undergone selection by decreasing disease sensibility to pathogenic fungi (Kono et al., 2018). If the same gene controls both processes, or both genes are very closely located, selection for one phenotype may have led to unwanted consequences for the other (selective sweep). Interestingly, it is observed that hairy grapevines are more often grown in colder climates whereas glabrous genotypes in hotter ones (Gago et al., 2016). Both pathogen susceptibility, which is more likely to cause disease in wetter climates, or drought may influence this dispersion of grapevine varieties. In future research it will be interesting to see if variation at the MYBF2 locus increased drought tolerance of grapevine. As transformation may be time-consuming, somatic mutants with a different hairiness maybe used in this perspective. At the moment, we are awaiting the results of a somatic mutant of Grenache, Lledoner Pelut, that may carry a mutation in this gene because the somatic variant has both prostrate trichomes and cuticular nanoridges in contrast to Grenache. Yet somatic variants may carry other mutations in their genome influencing the phenotype. Therefore, targeted mutagenesis techniques as CRISPR-CAS are currently developed for grapevine that will help to elucidate the effect of a single mutation on the phenotype.

Future developments for high throughput studies of diel transpiration patterns

To our knowledge, we developed and run the first high-throughput phenotyping protocol that allows to follow diel stomatal movements in controlled conditions. Moreover, this protocol can be used to investigate the effect of environmental changes such as light, CO₂ and VPD on diel stomatal dynamics. It's not applicable to leaves of plants experiencing drought as it is expected that leaves will rehydrate and show a different stomatal behaviour from leaves still attached to plant under dry conditions. However, different chemicals such as polyethylene glycol (PEG) mimicking drought, different pH, various pathogen elicitors or plant hormones could be put into the solution to observe the response of stomata on a 24-hours basis in combination with environmental conditions. The high-throughput capacity makes it possible to screen for genetic variation in stomatal responses to a range of external and internal cues.

Moreover, this protocol can be improved as the PHENOPSIS robot in the climate chamber is highly modular. Most exciting would be to combine stomatal behaviour with photosynthetic activity to know how diel stomatal patterns are coordinated with photosynthesis. This may be done on a high-throughput level by using chlorophyll reflectance (Flood et al., 2016; Genty and Meyer, 1995). In order to have a better proxy of stomatal behaviour in Arabidopsis, it is essential to be able to follow growth patterns during day and night, preferably using a 3D model to monitor the leaf movements which could participate in the variation of nighttime transpiration. Also for grapevine this could be advantageous: a 3D model of a curly leaf in the climate chamber could provide information about the light interception efficiency of different leaf morphology that could also be linked with stomatal dynamics. 3D models of plants can be obtained by using multiple cameras from different angles or using laser scanning techniques (Dornbusch et al., 2012). Furthermore, climate regulation may need to be optimized in order to have more stable and homogenous conditions for measurements. Monitoring of weight losses demands adaptations to the climate chamber which is initially designed to follow plant growth during its growth cycle. For example, humidification is not homogeneous in the chamber and

is dependent on the atmospheric pressure in the building. Depending on the pressure, thicker or smaller water particles were sent into the chamber, causing sometimes even the humidification of leaves (that we tried to correct or disregard). This was dramatic for monitoring weight losses, especially at night when weight losses were small and a small deposit of water could lead to unusable data. Also, we may optimize the speed of the automaton to be able to have a higher frequency of measurement in time. This can be advantageous to improve estimation of stomatal responses to environmental changes, but requires also a more stable environment for the scale. Turbulence in the chamber influenced the measurement of weight losses, causing especially problems for plants or leaves losing water at the limit of the range of the scale. In principle the scale is under the pots and does not have to be influenced by the turbulent conditions in the chamber. The advantage of a moving scale (rather than moving plants) is that no mechanical stress is induced in the plants, and that we have space to optimize the speed of the scale, while with moving plants limits the speed rate of weighing, notably when avoiding mechanical stress. In the future, the climate chamber is also expected to be equipped with LED lights allowing the responses of plants or leaves to different intensities of blue and red light that could help dissecting some physiological processes mentioned in this study.

This PhD aimed to identify the controllers of nighttime transpiration in grapevine. Contrary to our hypothesis, the locus explaining 15% of the variation in nighttime transpiration in grapevine was genetically linked to prostrate trichome density and the appearance of cuticular nanoridges but not to stomatal density or opening. A candidate gene and possible physiological mediators have been identified which shed a new light on the importance of cuticular aspects on water losses by plants and should participate in future development of water-use efficient varieties.

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Résumé (French)

L'approvisionnement en eau est essentiel pour soutenir la croissance des plantes, qui se développent à partir des assimilats issus de la fixation du CO₂ lors de la photosynthèse. Les pores stomatiques accélèrent la photosynthèse en facilitant la diffusion du CO₂ dans la feuille, mais au détriment de la vapeur d'eau perdue dans l'atmosphère lors de la transpiration. Lors d'un stress hydrique, la faible disponibilité de l'eau dans les feuilles ferme les stomates pour éviter une déshydratation complète de la feuille. En conséquence, la diffusion du CO₂ est entravée, ce qui réduit la photosynthèse et potentiellement la croissance. Si l'agriculture devient la plus grande consommatrice d'eau douce au monde, cela n'empêche pas la sécheresse de provoquer les plus fortes pertes de rendement dans l'agriculture. On prévoit que la demande en eau pour la production alimentaire doublera à l'avenir, alors que la disponibilité en eau diminue en raison du changement climatique et des besoins d'une population humaine croissante, ce qui souligne la nécessité d'utiliser l'eau plus efficacement dans l'agriculture. Les plantes présentent une variation considérable de la quantité d'eau nécessaire à leur croissance, et la compréhension de l'utilisation de l'eau par les plantes peut aider à produire des cultures à haut rendement et à utilisation efficace de l'eau. La viticulture couvre une grande partie des terres arables dans la région méditerranéenne. Toutefois, le maintien de cette culture emblématique dans un environnement aussi exposé à la sécheresse est gravement menacé par le changement climatique. L'exploration de grandes collections génétiques peut aider à trouver des cultivars qui utilisent moins d'eau. Parmi les cibles de sélection, une faible transpiration nocturne s'est récemment révélée pertinente, tant que l'assimilation du carbone pendant la journée, qui comme la transpiration est contrôlée par les pores stomatiques à la surface des feuilles, n'est pas réduite simultanément.

Le comportement dynamique des stomates dans des conditions environnementales stables suggère que l'ouverture stomatique est régulée par des processus endogènes. Les dynamiques stomatiques diurnes et nocturnes peuvent augmenter les pertes d'eau "inefficaces" en raison de l'absence apparente de coordination avec les gains de carbone. Dans cette perspective, cette étude a exploré les bases génétiques et physiologiques de la dynamique journalière (diurne et nocturne) de la transpiration chez la vigne (*Vitis vinifera*). Nous présentons ici une méthode à haut débit pour enregistrer la transpiration par gravimétrie dans des conditions contrôlées. Un robot de phénotypage pour la croissance d'*Arabidopsis thaliana* a été adapté pour enregistrer le poids de 150 individus toutes les 30 minutes à l'aide d'une balance mobile. De plus, nous avons développé un pipeline automatisé pour nettoyer les données de poids et pour dériver une dynamique de transpiration journalière fiable avec une résolution temporelle de 30 minutes. La dynamique de la transpiration peut être utilisée comme une approximation de l'ouverture des stomates à condition que des facteurs tels que les conditions environnementales, la croissance et les mouvements des feuilles soient pris en compte. Cette méthode a été appliquée sur des plantes entières de l'espèce modèle *Arabidopsis thaliana* et sur des feuilles détachées de *Vitis vinifera*, une espèce de culture de grand intérêt économique menacée par le changement climatique. L'analyse sur feuilles détachées présente plusieurs avantages : elle nécessite peu d'espace, ce qui permet d'obtenir davantage de réplicats, et le comportement stomatique peut être instantanément mesuré sur des feuilles matures ayant terminé leur croissance. Cela confère des avantages importants par rapport aux mesures effectuées sur des plantes entières à l'aide de capteurs de flux de sève, ou sur des plantes dont la distribution incontrôlée de l'âge des feuilles, la croissance des jeunes feuilles, les mouvements des feuilles peuvent masquer le comportement stomatique. Les feuilles de vigne détachées ont montré une étroite similitude avec la dynamique de transpiration des plantes entières et des feuilles attachées mesurée par échanges gazeux pendant 24 heures. De plus, notre méthode nous a permis de détecter une variation génétique pour le niveau de fermeture des stomates dans l'après-midi. Cette méthode a permis des études d'association à l'échelle du génome (GWAS) sur un panel de diversité composé de 279 cultivars, et

également des approches de gènes candidats chez *Arabidopsis thaliana*. Les analyses génétiques quantitatives ont révélé plusieurs polymorphismes nucléotidiques uniques (SNP) associés à différents aspects de la dynamique de transpiration, et l'exploration des bases de données génomiques et transcriptionnelles a mis en évidence plusieurs gènes candidats.

La génétique quantitative de la dynamique de la transpiration chez la vigne nous a permis d'identifier un gène candidat pour la transpiration diurne. Ce candidat, *MEX1*, code pour un transporteur de maltose essentiel pour le métabolisme nocturne de l'amidon. Le phénotypage du mutant *Arabidopsis mex1* a révélé qu'il est altéré dans l'ouverture stomatique induite par la lumière et la réouverture nocturne endogène. La comparaison de *mex1* avec d'autres mutants de la voie de l'amidon a indiqué que le rôle de *MEX1* dans le contrôle des mouvements stomatiques s'étend au-delà du métabolisme de l'amidon, mais nous n'avons pas pu conclure si son action est autonome dans les cellules de garde. En fin de compte, *MEX1* est susceptible d'avoir un effet important mais indirect sur l'homéostasie des sucres et de l'énergie dans les cellules de garde, qui régulent les processus de transport des solutés qui entraînent l'ouverture des stomates. La dissociation du métabolisme de l'amidon et des mouvements stomatiques offre une nouvelle cible pour la sélection de plantes avec une meilleure efficacité d'utilisation de l'eau.

Nous avons également trouvé une association inattendue pour la transpiration nocturne avec l'abondance de trichomes couchés et de nano-stries cuticulaires sur l'épiderme des feuilles de vigne. La morphogenèse des tissus crée des patrons de structures cellulaires avec des fonctions dédiées dans leur environnement dominant. À l'interface avec l'atmosphère, l'épiderme des feuilles de plantes est constitué de cellules « de pavage » non spécialisées, qui sont entrecoupées de valves bicellulaires appelées stomates et d'excroissances appelées poils ou trichomes. Pour s'adapter à l'atmosphère sèche lors de la colonisation des terres il y a 500 millions d'années, les plantes ont développé une couche hydrophobe de cuticule faite de cutine et de cire qui recouvre les cellules épidermiques, agissant comme une barrière contre la perte d'eau. La composition et la morphologie de la cuticule des feuilles sont extrêmement variables d'une espèce à l'autre et au sein d'une même espèce, mais il est difficile de savoir dans quelle mesure les modifications des propriétés de la cuticule affectent la capacité d'imperméabilisation de l'épiderme. Nous montrons ici que la perte d'eau nocturne chez la vigne est congruente avec la morphogenèse de l'épiderme des feuilles, probablement contrôlée par le facteur de transcription MYBF2. L'analyse de la transpiration nocturne dans un panel d'association de la vigne a révélé un locus principal qui co-localise avec une association pour les trichomes couchés et les nano-stries sur la cuticule épidermique. Des analyses plus approfondies ont permis d'étayer un modèle dans lequel le candidat sous-jacent MYBF2 spécifie le développement à la fois de trichomes prostrés et de nano-stries cuticulaires, ces derniers reflétant des changements plus profonds dans la cuticule résultant en une augmentation de la perte d'eau. MYBF2 appartient à une sous-famille de R2R3-MYBs normalement impliqués dans le métabolisme secondaire, également responsable de la couleur rose de la tomate. Nous proposons que la néofonctionnalisation de MYBF2 a été sélectionnée lors de la domestication de la vigne. Alors que les autres MYBs de son sous-clade sont spécialisés dans le métabolisme des flavonoïdes, nos données suggèrent que MYBF2 a acquis de nouvelles fonctions liées à la morphogenèse épidermique. Il est tentant de spéculer que la production de trichomes – le seul caractère visible à l'œil nu – a été sélectionnée en premier comme barrière contre les agents pathogènes. En accord avec cette proposition, un QTL pour les trichomes couchés, correspondant à notre SNP sur le chromosome 5, co-localise avec un QTL de résistance au mildiou. Néanmoins, les propriétés de la cuticule affectent également d'autres aspects de la défense des plantes de plusieurs façons, par exemple par la détection des pathogènes (Bessire et al 2007 EMBO J) ou la transmission de la résistance systémique acquise. Ainsi, les nano-stries cuticulaires peuvent également contribuer d'une manière ou d'une autre à améliorer la défense de la vigne. Dans le contexte du changement

climatique, notre étude ouvre la voie à la conception de stratégies visant à découpler les différentes facettes de la morphogenèse épidermique afin de réduire l'effet « incontinent » de MYBF2 sur la transpiration nocturne, tout en conservant ses propriétés de défense.

Nous avons développé et mis en œuvre un protocole de phénotypage à haut débit qui permet de suivre les mouvements stomatiques dans des conditions contrôlées. De plus, ce protocole peut être utilisé pour étudier l'effet des changements environnementaux tels que la lumière, le CO₂ et le VPD sur la dynamique stomatique. Il n'est pas directement applicable aux feuilles détachées issues de plantes en situation de sécheresse, car on s'attend à ce que les feuilles se réhydratent et présentent un comportement stomatique différent de celui des feuilles encore attachées à la plante dans des conditions sèches. Cependant, différents produits tels que le polyéthylène glycol (PEG) ou mannitol imitant la sécheresse, un pH différent, divers éliciteurs d'agents pathogènes ou des hormones végétales pourraient être mis dans la solution pour observer la réponse des stomates sur une base de 24 heures en combinaison avec les conditions environnementales. La capacité à haut débit permet de détecter la variation génétique des réponses stomatiques à une série de stimuli externes et de contrôles internes.

De plus, ce protocole peut être amélioré car le robot PHENOPSIS de la chambre climatique est très modulaire. Le plus intéressant serait de combiner les analyses du comportement stomatique avec les échanges gazeux pendant le jour et la nuit pour rechercher les liens possibles entre les stomates et la photosynthèse pendant le jour et la nuit et vice-versa. Afin de disposer d'une meilleure approximation du comportement stomatique chez *Arabidopsis*, il est utile de pouvoir suivre les dynamiques de croissance en parallèle pendant le jour et la nuit, de préférence en utilisant la 3D pour surveiller les mouvements des feuilles qui pourraient participer à la variation de la transpiration nocturne. Cela pourrait également être avantageux pour la vigne : un modèle 3D d'une feuille (qui présente souvent des ondulations chez la vigne) dans la chambre climatique pourrait fournir des informations sur l'efficacité d'interception de la lumière qui pourrait également être liée à la dynamique stomatique. Les modèles 3D des plantes peuvent être obtenus en utilisant plusieurs caméras sous différents angles ou en utilisant des techniques de balayage laser. En outre, la régulation climatique pourrait devoir être optimisée afin d'avoir des conditions plus stables et homogènes pour les mesures. Le suivi des pertes de poids a exigé des adaptations de la chambre climatique qui était initialement conçue pour suivre la croissance des plantes pendant leur cycle. Par exemple, l'humidification n'est pas homogène dans la chambre et dépend de la pression du réseau d'air comprimé dans le bâtiment. En fonction de la pression, des particules d'eau plus ou moins épaisses ont été envoyées dans la chambre, provoquant parfois même l'humidification des feuilles (que nous avons essayé de prendre en compte). Cette situation était problématique pour le suivi des pertes de poids, surtout la nuit, lorsque les pertes de poids étaient faibles et qu'un petit dépôt d'eau pouvait conduire à des données inutilisables. De plus, nous pouvons optimiser la vitesse de l'automate pour pouvoir avoir une fréquence de mesure plus élevée dans le temps. Cela peut être avantageux pour améliorer l'estimation des réponses stomatiques aux changements environnementaux, mais cela nécessite également un environnement plus stable pour la balance. Les turbulences dans la chambre ont influencé la mesure des pertes de poids, causant notamment des problèmes pour les feuilles perdant de l'eau avec une vitesse à la limite de la précision de la balance pour notre pas de temps. En principe, la balance se trouve sous les pots et ne doit pas être influencée par les conditions dans la chambre. L'avantage d'une balance mobile (plutôt que des plantes en mouvement) est qu'aucun stress mécanique n'est induit dans les plantes, et que nous avons de la place pour optimiser la vitesse de la balance, alors qu'avec des plantes en mouvement, la vitesse de pesée doit être limitée. À l'avenir, la chambre climatique devrait également être équipée de LED permettant de manipuler la quantité de lumière bleue et rouge, ce qui pourrait également aider à disséquer certains processus physiologiques mentionnés dans cette étude.

Ce doctorat visait à identifier les contrôleurs de la transpiration nocturne de la vigne. Contrairement à notre hypothèse, ce ne sont pas les stomates mais les caractères cuticulaires qui influencent le plus la variation génétique de la transpiration nocturne dans nos conditions. Le polymorphisme génétique sous-jacent et les acteurs moléculaires ont été identifiés pour un locus, ce qui jette un nouvel éclairage sur l'importance des aspects cuticulaires sur les pertes d'eau par les plantes et devrait participer au développement futur de variétés efficaces en termes d'utilisation de l'eau. Pour en revenir à la question de savoir si les niveaux élevés de transpiration nocturne chez *Arabidopsis thaliana* sont dus à une « fuite » stomatique ou à une transpiration cuticulaire, on pourrait spéculer sur le rôle de l'ABA. Les expériences d'ABA sur des feuilles détachées de Col-0 ont montré une fermeture marginale des stomates dans l'obscurité et nous amènent à l'hypothèse qu'une transpiration cuticulaire élevée provoque une transpiration nocturne. Cependant, l'effet marginal de l'ABA sur la conductance des feuilles la nuit peut être causé par une sensibilité moindre des stomates à l'ABA, et donc entraîner une fermeture stomatique moindre.

La diminution de la transpiration nocturne chez les cultivars de vigne peut être un moyen élégant d'améliorer l'efficacité de l'utilisation de l'eau par les vignes. Notre protocole utilise des feuilles de vigne cultivées à l'extérieur qui sont plus représentatives des conditions du terrain que les feuilles cultivées en serre. De plus, les niveaux de transpiration des plantes entières à l'extérieur étaient similaires à ceux observés sur des feuilles détachées dans la chambre. Cependant, la contribution de la transpiration nocturne à la transpiration totale pendant la journée était plus faible dans le champ que dans la chambre climatique, en raison du rayonnement relativement faible dans la chambre climatique. Nos résultats indiquent que la transpiration nocturne ne peut contribuer qu'à une petite partie de la transpiration diurne totale. Cependant, les caractéristiques liées à la cuticule devraient également influencer l'utilisation de l'eau pendant la journée, ce qui indique qu'en utilisant le QTL détecté pour cette caractéristique, nous pouvons économiser de l'eau sur le cycle journalier total (bien qu'on n'ait pas détecté ce SNP en transpiration diurne ou journalière, sans doute car l'effet est trop faible par rapport à la composante stomatique en journée), et a fortiori en conditions de stress hydrique où la contribution relative de la cuticule devient plus importante. On pourrait économiser encore plus d'eau à condition que les caractéristiques des trichomes puissent être sélectionnées indépendamment de la cuticule. En effet, un plus grand nombre de trichomes devrait diminuer à la fois l'échange d'eau et de CO₂ pendant la journée, tandis qu'une cuticule plus épaisse peut permettre d'économiser de l'eau sans altérer la photosynthèse. Dans l'idéal, il serait intéressant de combiner les effets positifs de ces deux caractéristiques qui dépendront des conditions locales. Une diminution des pertes d'eau pendant la nuit peut permettre d'économiser l'eau du sol qui peut être utilisée plus tard dans la saison sèche pour assurer la maturation et garantir un rendement minimal. L'effet d'une variété à faible transpiration nocturne a été modélisé dans une étude sur le bilan hydrique des vignobles que nous avons publiée dans la Revue des Œnologues. Cette étude a montré que, si l'on économise davantage d'eau la nuit, celle-ci sera utilisée pendant la journée et pourrait entraîner une fixation plus importante du carbone. Cela peut ne pas être à l'avantage du cépage économiseur d'eau tant que la disponibilité de l'eau est maintenue à un niveau élevé. Cependant, dans la seconde moitié du cycle de la plante, pendant la maturation, le sol s'assèche généralement à des niveaux bas, ce qui limite l'ouverture des stomates pendant la journée. La transpiration nocturne représente alors une part croissante des pertes d'eau sur 24 heures, et une variété à transpiration nocturne réduite à ce stade peut offrir un avantage substantiel pour retarder une déshydratation sévère du sol qui bloque la maturation. Dans l'ensemble, un gène ou une combinaison de gènes réduisant la transpiration nocturne peut être bénéfique à deux égards : le report d'un assèchement grave du sol et l'augmentation de l'eau disponible pour maintenir l'échange gazeux. Une autre différence majeure avec le terrain est que les vignes sont greffées sur un porte-greffe pour éviter l'infection du phylloxéra

(*Daktulosphaira vitifoliae*). Il a été démontré que le porte-greffe a un effet significatif sur la transpiration du greffon, qui est régulée par des signaux chimiques et hydrauliques. Ceci est probablement lié à la production d'ABA par les racines qui est transportée vers les feuilles et induit une fermeture stomatique. On ne sait pas dans quelle mesure le porte-greffe pourrait modifier les résultats que nous avons obtenus sur des plants francs-de-pied, en feuilles détachées de surcroît. La corrélation observée entre la transpiration nocturne des feuilles détachées et des plantes entières sur un petit ensemble de génotypes et pour une certaine variation environnementale (CO₂ ambiant et élevé) suggère que la plus grande partie de la variation est conservée en présence d'un système racinaire, mais nous n'avons pas fait varier le génotype de la partie racinaire, ce qui reste à explorer.

Dans notre étude, nous n'avons pas constaté de forte corrélation entre la transpiration diurne et nocturne, comme observé auparavant. Cela peut être dû aux différentes conditions de croissance et aux protocoles utilisés pour mesurer la transpiration (feuilles détachées - plante entière). La sélection pour un caractère cuticulaire peut être avantageuse en raison du faible impact sur l'échange gazeux diurne et sur la photosynthèse. Cependant, la production de lipides peut nécessiter plus d'énergie de la part de la plante. Un bilan coûts-avantages doit être établi pour savoir dans quelles conditions nous avons besoin de plantes qui présentent une conductance cuticulaire plus faible. L'impact d'un caractère cuticulaire pour réduire la transpiration pendant la nuit peut être d'un intérêt majeur dans les périodes de fermeture stomatique induite par la sécheresse. À l'heure actuelle, nous ne savons pas si la domestication a conduit à la sélection de génotypes de vigne ayant une cuticule plus ou moins perméable. Si ce caractère est moins important en période de sécheresse, il se peut qu'il n'ait pas été sélectionné. Les trichomes peuvent avoir fait l'objet d'une sélection parce qu'ils confèrent une moindre sensibilité à la maladie. Si le même gène contrôle les deux processus, ou si les deux gènes sont très proches (balayage sélectif), la sélection pour un phénotype peut avoir entraîné des conséquences indésirables pour l'autre. Il est intéressant de noter que les vignes poilues sont plus souvent cultivées dans des climats froids alors que les génotypes glabres sont cultivés dans des climats plus chauds. La sensibilité aux pathogènes (qui est plus susceptible de provoquer des maladies dans les climats plus humides) ou la sécheresse peuvent influencer cette dispersion des variétés de vigne. Dans les recherches futures, il sera intéressant de voir si les vignes en conditions de terrain bénéficient d'allèles favorables de notre gène candidat. Comme la transformation peut prendre du temps, des mutants somatiques avec une pilosité différente peuvent être utilisés dans cette perspective. Des techniques CRISPR-CAS sont également développées actuellement pour être appliquées à la vigne, ce qui pourrait potentiellement stimuler la recherche sur les fonctions des gènes dans le domaine.

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Lotta, you have been the most special person that I have met in my life. I am very grateful having you close and I dedicate this thesis to you.

About the Author

Adrianus Johannes Westgeest was born on 25 March 1993 in 's Gravenhage, The Netherlands. He attended the “Adelbert College” in Wassenaar, from which he graduated in 2011. In the same year he started to study Plant Sciences at Wageningen University. For his first thesis, he conducted research at the Abiotic Stress group under supervision of dr. G van der Linden and dr. J.R. Chan Navarette at the Plant Breeding department (Wageningen University). This resulted in a bachelor thesis entitled: “Nitrogen Use Efficiency in spinach”. He continued his Master in Plant Biotechnology with the specialization Molecular Breeding and Pathology. In his masters he went first abroad to the department of Genetics (Max Planck Institute for plant breeding research, Germany) for a minor-thesis under supervision of prof. dr. M. Koornneef and dr. W. van Esse. This thesis was entitled: “The genetics and morphological analysis of the barley low-tillering mutant *intermedium-b*”. Afterwards he did his MSc thesis under supervision of prof. dr. R. van der Vlugt at the department of Virology at Wageningen University. This work was presented on a poster at a scientific conference entitled: “Virus infection by PepMV increases drought tolerance in *Nicotiana benthamiana*”. For his MSc internship he went to the LEPSE (Montpellier, France) to work on a report entitled: “Effects of abscisic acid on leaf hydraulics in *Vitis vinifera*” under supervision of dr. F. Pantin and dr. T. Simonneau. He graduated for his MSc in 2017 at Wageningen University. Afterwards he worked as a scientific engineer at LEMP-CNRS CEA Cadarache (St-Paul-lez-Durance, France) with dr. B. Genty. Before starting his PhD in September 2017, he worked as a scientific engineer at the LEPSE with dr. F. Pantin on leaf hydraulics in grapevine and participated successfully in the “Concours Ecole Doctorale GAIA 2017”.



Education Statement graduate school GAIA, Montpellier

Publications

Pantin F, Coupel-Ledru A, Westgeest A, Simonneau T. (2019) Adaptation au changement climatique. Sélectionner des vignes qui transpirent moins la nuit. *Revue des OEnologues* 172: Juillet 2019

Westgeest AJ, Cortello J, Pantin F, Simonneau T, A puzzling role for MALTOSE EXCESS 1 in stomatal dynamics (in preparation)

Westgeest AJ, Coupel-Ledru A, Flutre T, Doligez A, This P, Gago P, Pantin F, Simonneau T, Genetic control of nighttime transpiration through MYB-specified epidermal sophistication in grapevine (in preparation)

Post-Graduate Courses

Ethique et intégrité en science, 7 hours (University of Montpellier, 2018)

Papirus: publier et communiquer en science, 25 hours (INRA Virtuel Class, 2018)

GAIA meetings

Journée rentrée solennelle (2018)

Teaching

Practical assistant grapevine ecophysiology at SupAgro, 8 hours (May-2018)

Practical assistant TP Phenopsis, 8 hours (December-2018)

Participation “Fête de la science Occitanie”, 3 hours (October-2018)

Poster presentations at international conferences

Mizokami Y, Westgeest A, Reiter I, Pantin F, Genty B (2017) Effects of ABA, CO₂ and VPD on leaf hydraulics. *Xylem Group Annual Meeting*, Bordeaux, France

Westgeest A, Cortello J, Conéjéro G, Simonneau T, Pantin F (2017) Effects of abscisic acid on leaf hydraulics in *Vitis vinifera*. *Xylem Group Annual Meeting*, Bordeaux, France

Oral presentations

The control of nighttime stomatal kinetics, internal lab seminar Narco-Lepsie, Montpellier (April-2019)

Quantitative genetics of stomatal kinetics in grapevine, AGAPIADES PhD students, Montpellier (May-2019)

Variation génétique des dynamiques de transpiration, Workshop : Changement Climatique, Eau, Vigne et Vin – projet Laccave 2.21, Gruissan (Oct-2019)

Personal development

Conference of the “parcours doctorale EIR-A” organised by Agreenium (Lyon, March-2018)

Organisation of the “journée des doctorants for post-docs and PhD students working on wine and vine research in Montpellier”, for KIM MUSE Montpellier with Elise Bourru (September-2019)

Set-up and organisation of the weekly internal lab seminar with Stéphane Leveau et Sibylle Dueri (2018-2020)

Funding & Supervision

The research in this project was carried at the UMR LEPSE, Montpellier, France, supervised by dr. F. Pantin (SupAgro Montpellier) and dr. T. Simonneau (INRA Montpellier). This project was financially supported by the Region Occitanie (France) and INRAE. The comité de suivi was consisting of dr. P. Martre (INRA Montpellier), dr. B. Lacombe (CNRS Montpellier), dr O. Brendel (INRA Nancy), dr. A. Doligez (INRA Montpellier) and dr. L. Le Cunff (IFV).

Contact

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Abstract

Viticulture covers a large part of arable lands in the Mediterranean regions. However, maintaining this emblematic crop in such drought-prone environments is critically challenged by climate change. Exploring large genetic collections can help to breed cultivars with enhanced water use efficiency. Leaf stomata, which are tiny epidermal pores delineated by guard cells that can open and close, play a pivotal role in this process as they regulate gas exchange – photosynthesis and transpiration – between the plant and the atmosphere. Due to stomatal behaviour, enhanced photosynthesis generally coincides with enhanced transpiration. Accounting for this process, lowering transpiration at night has been recently proposed as a promising breeding strategy to reduced water loss without penalty on photosynthesis. However, daytime and nighttime transpiration are interrelated to some extent. Here, we explored the genetic and physiological bases of diel (24-h) dynamics of transpiration in grapevine (*Vitis vinifera*). A pipeline was developed for high-throughput phenotyping of transpiration dynamics on detached leaves. This enabled genome wide association studies (GWAS) on a diversity panel made of 279 cultivars, which generated candidate genes that were further addressed by different approaches. Quantitative genetic analyses revealed several single nucleotide polymorphisms (SNPs) associated with different aspects of the diel transpiration dynamics. Notably, we found an association for nighttime transpiration that co-localized with the abundance of prostrate trichomes and cuticular nanoridges on the leaf epidermis. A MYB transcription factor was identified as a good candidate involved in the production of both structures, showing a complex interplay with stomatal development. Based on genomic and coexpression analyses, we propose a new model of interacting genes involved in the development of epidermal structures and impacting nighttime transpiration. Unexpectedly, we also found an association for daytime transpiration that was close to a gene that is essential for nighttime starch metabolism. The corresponding mutant in *Arabidopsis thaliana* was impaired in light-induced stomatal opening and endogenous nighttime reopening. Comparison with other mutants of the pathway revealed that the role of this gene in controlling stomatal movements extends beyond starch metabolism. Its cell-autonomous action was addressed, without unequivocal conclusion. Overall, this work provided several targets of interest to save water at night and favouring higher water use efficiency in grapevine.

Abstract

La viticulture couvre une grande partie des terres arables dans les régions méditerranéennes. Cependant, le maintien de cette culture emblématique dans des environnements sujets à la sécheresse est menacé par le changement climatique. L'exploration de larges collections génétiques peut aider à sélectionner des cultivars plus efficaces en termes d'utilisation de l'eau. Les stomates, de minuscules pores sur l'épiderme des feuilles délimités par des cellules de garde pouvant s'ouvrir et se fermer, jouent un rôle essentiel dans ce processus car ils régulent les échanges gazeux – photosynthèse et transpiration – entre la plante et l'atmosphère. En raison du comportement stomatique, une photosynthèse accrue coïncide généralement avec une transpiration accrue. Ainsi, réduire la transpiration nocturne apparaît comme une stratégie de sélection prometteuse pour réduire les pertes en eau sans pénaliser la photosynthèse. Cependant, la transpiration diurne et nocturne sont corrélés dans une certaine mesure. Ici, nous avons exploré les bases génétiques et physiologiques de la dynamique journalière (24 h) de la transpiration chez la vigne (*Vitis vinifera*). Un protocole a été développé pour le phénotypage à haut débit de la dynamique de la transpiration sur feuilles détachées. Cela a permis de réaliser des études d'association pangénomique (GWAS) sur un panel de diversité composé de 279 cultivars, ayant généré des gènes candidats qui ont ensuite été étudiés par différentes approches. Des analyses génétiques quantitatives ont révélé plusieurs polymorphismes nucléotidiques uniques (SNP) associés à différents aspects de la dynamique de la transpiration. Nous avons notamment trouvé une association pour la transpiration nocturne qui co-localise avec l'abondance de trichomes couchés et de stries cuticulaires sur l'épiderme des feuilles. Un facteur de transcription MYB a été identifié comme un candidat probant impliqué dans la production des deux structures, montrant une interaction complexe avec le développement stomatique. Sur la base d'analyses génomiques et de coexpression, nous proposons un nouveau modèle de gènes en interaction, impliqués dans le développement des structures épidermiques et ayant un impact sur la transpiration nocturne. De manière inattendue, nous avons également trouvé une association pour la transpiration diurne proche d'un gène essentiel au métabolisme nocturne de l'amidon. Le mutant correspondant chez *Arabidopsis thaliana* était altéré dans l'ouverture stomatique à la lumière et la réouverture nocturne endogène. La comparaison avec d'autres mutants de la voie a révélé que le rôle de ce gène dans le contrôle des mouvements stomatiques s'étend au-delà du métabolisme de l'amidon. Son action cellule-autonome a été étudiée, avec des conclusions équivoques. Dans l'ensemble, ces travaux ont fourni plusieurs cibles intéressantes pour économiser l'eau la nuit et favoriser une meilleure efficacité d'utilisation de l'eau chez la vigne.